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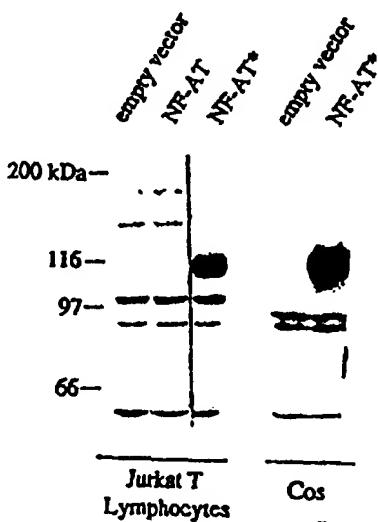
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| (51) International Patent Classification 7 :<br><br>A61K 38/00   | A2 | (11) International Publication Number: <b>WO 00/30671</b><br><br>(43) International Publication Date: 2 June 2000 (02,06,00)  |
| (21) International Application Number: PCT/US99/27862  |    | (81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). |
| (22) International Filing Date: 23 November 1999 (23.11.99)  |    |   |
| (30) Priority Data:<br>09/198,977 24 November 1998 (24.11.98) US   |    | Published<br><i>Without international search report and to be republished upon receipt of that report.</i>                    |
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(54) Title: NF-AT MEDIATES CARDIAC HYPERTROPHY, METHODS AND REAGENTS RELATED THERETO



(57) Abstract

The invention is a method for treating cardiac hypertrophy, or other preventing other growth of cardiac and vascular tissue, through the use of NF-AT antagonists.

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***NF-AT Mediates Cardiac Hypertrophy,  
Methods and Reagents Related Thereto***

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**Government Support**

This invention was made in the course of work supported by the U.S. Government and Howard Hughes Medical Institute, which may have certain rights in this invention.

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**Background of the Invention**

Heart failure affects approximately three million Americans, developing at a rate of approximately 400,000 new cases per year. Current therapy for heart failure is primarily directed to using angiotensin-converting enzyme (ACE) inhibitors and diuretics. ACE inhibitors appear 15 to slow the progression towards end-stage heart failure in patients; however, they are unable to relieve symptoms in more than 60% of heart failure patients and reduce mortality of heart failure only by approximately 15-20%. Heart transplantation is limited by the availability of donor hearts. With the exception of digoxin, the chronic administration of positive inotropic agents has not resulted in a useful drug without accompanying adverse side effects, such as increased arrhythmia, 20 sudden death, or other deleterious side effects related to survival. These deficiencies in current therapy suggest the need for additional therapeutic approaches.

Cardiac muscle hypertrophy is one of the most important adaptive physiological responses of the myocardium. In response to increased demands for cardiac work or following a variety of pathological stimuli which lead to cardiac injury, the heart adapts through the activation of a 25 hypertrophic response in individual cardiac muscle cells, which is characterized by an increase in myocyte size, the accumulation of contractile proteins within individual cardiac cells, the activation of embryonic gene markers expression, and the lack of a concomitant effect on muscle cell proliferation. Although the hypertrophic process can initially be compensatory, there can be a pathological transition in which the myocardium becomes dysfunctional (Braunwald (1994) in 30 Pathophysiology of Heart Failure, (Braunwald, ed.); Saunders, Philadelphia; Vol. 14, pp 393-402).

Studies in an in vitro model system of ventricular muscle cell hypertrophy have led to the identification of a number of mechanical, hormonal, growth factor, and pathological stimuli which can activate several independent features of hypertrophy (Chien et al. (1991) FASEB J. 5:3037-3046; Knowlton et al. (1991) J. Biol. Chem. 266:7759-7768; Shubeita et al. (1990) J. Biol. Chem. 35 265:20555-20562; Thorburn et al. (1993) J. Biol. Chem. 268:2244-2249; LaMorte et al. (1994)

J. Biol. Chem. 269:13490-13496; Knowlton et al. (1993) J. Biol. Chem. 268:15374-15380). Currently, there are at least two signal transduction pathways, involving both ras- (Thorburn et al. (1993) *supra*), and G<sub>q</sub> protein-dependent downstream effectors (LaMorte et al. (1994) *supra*) implicated in the activation of features of the hypertrophic response in the in vitro model system.

5 While a great deal of progress has been made in uncovering the signaling pathways which activate the ventricular muscle cell hypertrophic response, relatively little has been reported as to the mechanisms which might inhibit or suppress the hypertrophic response in a non-lethal manner.

10 There is a clear need for new drugs to treat cardiac disorders, e.g., to improve heart failure therapy, such as congestive heart failure and hypertrophic cardiomyopathy. Methods for identifying such drugs are thus necessitated.

### **Summary of the Invention**

15 One aspect of the present invention provides a method for preventing or reducing cardiac hypertrophy in a subject, comprising administering to the subject a pharmaceutically effective amount of an NF-AT antagonist to decrease the biological activity of NF-AT in myocardial tissue, to thereby prevent or reduce cardiac hypertrophy in the subject.

20 For example, the method of the present invention can use NF-AT antagonists which decrease the transcriptional activity of NF-AT, inhibit the nuclear translocation of NF-AT, and/or inhibits dephosphorylation of NF-AT.

25 In certain embodiments, the antagonists of the present invention inhibit the formation of a complex comprising NF-AT, e.g., a protein-protein or protein-DNA interaction. For example, the antagonist can inhibit an NF-AT activity by inhibiting interaction of the protein with a gene which includes an NF-AT responsive element, or it can inhibit dephosphorylation of an NF-AT protein, or inhibit nuclear localization of an NF-AT protein.

The antagonist can be delivered locally or systemically. In the instance of the former, the antagonist can be delivered by catheter, and/or perfusion into the myocardial space.

The present method can be used as part of a treatment for, e.g., congestive heart disease.

In preferred embodiments, the antagonist selectively inhibits NF-ATc3 and/or NF-ATc4.

30 The antagonist can be identified by a method comprising

- (i) contacting an isolated NF-AT polypeptide or portion thereof sufficient for interacting with a molecule, with the molecule and a compound in conditions under which, but for the presence of the compound, the NF-AT polypeptide or portion thereof and the molecule interact; and

5 (ii) determining the level of interaction between the NF-AT polypeptide or portion thereof and the molecule in the presence relative to the absence of the compound, such that a weaker interaction between the NF-AT polypeptide or portion thereof and the molecule in the presence, relative to the absence, of the compound indicates that the compound is an antagonist of the activity of an NF-AT polypeptide.

In still another aspect, the present invention provides a pharmaceutical composition for treating or preventing cardiac hypertrophy comprising an NF-AT antagonist in a pharmaceutically acceptable delivery vehicle for targeting the NF-AT antagonist to the heart.

10 The present invention also provides several novel methods and compositions for modulating the immune response and for screening for modulators of the immune response. These methods utilize polynucleotide sequences encoding NF-AT<sub>c</sub> recombinant proteins and complementary polynucleotides which are substantially identical to NF-AT<sub>c</sub> polynucleotide sequences.

15 In one aspect of the invention, NF-AT<sub>c</sub> polypeptides and compositions thereof are provided. NF-AT<sub>c</sub> polypeptides comprise polypeptide sequences which are substantially identical to a sequence shown in Fig. 1 or a cognate NF-AT<sub>c</sub> gene sequence.

20 Nucleic acid sequences encoding NF-AT<sub>c</sub> are provided. The characteristics of the cloned sequences are given, including the nucleotide and predicted amino acid sequence in Fig. 1. Polynucleotides comprising these sequences can serve as templates for the recombinant expression of quantities of NF-AT<sub>c</sub> polypeptides, such as human NF-AT<sub>c</sub> and murine NF-AT<sub>c</sub>. Polynucleotides comprising these sequences can also serve as probes for nucleic acid hybridization to detect the transcription and mRNA abundance of NF-AT<sub>c</sub> mRNA in individual lymphocytes (or other cell types) by *in situ* hybridization, and in specific lymphocyte populations by Northern blot analysis and/or by *in situ* hybridization (Alwine et al. (1977) Proc. Natl. Acad. Sci. U.S.A. 74: 5350) and/or PCR amplification and/or LCR detection. Such recombinant polypeptides and nucleic acid hybridization probes have utility for *in vitro* screening methods for immunomodulatory agents and for diagnosis and treatment of pathological conditions and genetic diseases, such as transplant rejection reactions, T cell-mediated immune responses, lymphocytic leukemias (e.g., T cell leukemia or lymphoma) wherein NF-AT activity contributes to disease processes, autoimmune disease, arthritis, and the like.

25 In one embodiment, candidate immunomodulatory agents are identified by their ability to block the binding of a NF-AT<sub>c</sub> polypeptide to other components of NF-AT (e.g., AP-1) and/or to block the binding of NF-AT to DNA having an NF-AT recognition site. The DNA preferably includes one or more NF-AT binding sites at which a NF-AT protein complex specifically binds.

One means for detecting binding of a NF-AT protein comprising NF-AT<sub>c</sub> to DNA is to immobilize the DNA, such as by covalent or noncovalent chemical linkage to a solid support, and to contact the immobilized DNA with a NF-AT protein complex comprising a NF-AT<sub>c</sub> polypeptide that has been labeled with a detectable marker (e.g., by incorporation of radiolabeled 5 amino acid). Such contacting is typically performed in aqueous conditions which permit binding of a NF-AT protein to a target DNA containing a NF-AT binding sequence. Binding of the labeled NF-AT to the immobilized DNA is measured by determining the extent to which the labeled NF-AT<sub>c</sub> polypeptide is immobilized as a result of a specific binding interaction. Such 10 specific binding may be reversible, or may be optionally irreversible if a cross-linking agent is added in appropriate experimental conditions.

In one aspect, candidate immunomodulatory agents are identified as being agents capable of inhibiting (or enhancing) intermolecular binding between NF-AT<sub>c</sub> and other polypeptides which comprise a NF-AT complex (e.g., AP-1, JunB, etc.). The invention provides methods and 15 compositions for screening libraries of agents for the capacity to interfere with binding of NF-AT<sub>c</sub> to other NF-AT polypeptide species under aqueous binding conditions. Typically, at least either NF-AT<sub>c</sub> and/or another NF-AT polypeptide species is labeled with a detectable label and 20 intermolecular binding between NF-AT<sub>c</sub> and other NF-AT polypeptide species is detected by the amount of labeled species captured in NF-AT complexes and the like.

Based at least in part on the observation that NF-AT polypeptides comprise nuclear 25 localization sequences, which allow NF-AT polypeptides to translocate to the nucleus in the presence of intracellular calcium, but which are shielded by forming intramolecular associations with other domains in the NF-AT polypeptide in the absence of calcium, the invention also provides methods for modulating the activity of NF-AT, by modulating translocation of NF-AT, such as by modulating intramolecular associations and shielding of a nuclear localization 30 sequence (NLS). In addition, since the NLS of NF-AT form an intramolecular association with a phosphorylated domain of NF-AT, the invention provides methods for modulating NF-AT activity, comprising modulating NF-AT phosphorylation. Furthermore, since, as disclosed herein, NF-AT is phosphorylated by protein kinase A (PKA) and glycogen synthase kinase-3 (GSK-3), and dephosphorylated by calcineurin, the state of phosphorylation and thus activation of NF-AT, can be modulated by modulating the activity of these kinases and/or phosphatase. Also within 35 the scope of the invention are compounds which modulate nuclear translocation of NF-AT, as well as screening assays for identifying additional compounds.

In one aspect, the present invention provides a method for identifying an agent that modulates phosphorylation of an NF-AT, comprising contacting test agent with a mixture including GSK-3 kinase activity with an NF-AT polypeptide, or portion thereof which is a substrate of the GSK-3 kinase activity, and determining the ability of the test agent to modulate 4

the interaction of the GSK-3 kinase activity with the NF-AT polypeptide and/or phosphorylation of the NF-AT polypeptide by the GSK-3 kinase activity.

The subject assay can be carried out in as a cell-based assay, e.g., employing a recombinant GSK-3 kinase, NF-AT substrate and/or reporter gene, or in a cell-free format, e.g., 5 using purified or semi-purified preparations of GSK-3 and the NF-AT substrate. The assay can be a simple competitive binding assay, or a kinase activity assay which detects the rate of phosphorylation of the substrate, or a nuclear translocation assay which detects the rate of nuclear localization of the substrate. In preferred embodiments, the subject method includes a further step 10 of formulating a pharmaceutical preparation including one or more compounds identified in the subject assay.

Another aspect of the present invention provides a method for modulating NF-AT phosphorylation, comprising contacting a cell expressing NF-AT with an agent that modulates the phosphorylation of NF-AT by GSK-3, e.g., using a GSK-3 inhibitor, especially an inhibitor which inhibits NF-AT nuclear translocation.

15 Still another aspect of the present invention relates to peptide or peptidomimetic agents for modulating nuclear translocation of an NF-AT protein, which agent corresponds to a portion of an NF-AT protein involved in intramolecular association of nuclear localization signals. The present invention also provides a method for identifying compounds that modulate nuclear 20 translocation of NF-AT, comprising contacting a test agent with a NF-AT polypeptide, or portion thereof which includes a nuclear localization signal, and determining the ability of the test agent to bind to the nuclear localization sequence and/or alter the tertiary structure of a phosphorylated form of the nuclear localization sequence. As above, such assays can be carried 25 out in cell-based and cell-free formats, e.g., as competitive binding assays or nuclear translocation assays. In one embodiment, changes in the conformation of the protein which are dependent on phosphorylation of the NLS sequences can be detected photometrically, such as by CD/ORD or other means for determining changes in tertiary structure

The invention also provides antisense polynucleotides complementary to NF-AT<sub>c</sub> sequences which are employed to inhibit transcription and/or translation of the cognate mRNA species and thereby effect a reduction in the amount of the respective NF-AT<sub>c</sub> protein in a cell 30 (e.g., a T lymphocyte of a patient). Such antisense polynucleotides can function as immunomodulatory drugs by inhibiting the formation of NF-AT protein required for T cell activation.

In a variation of the invention, polynucleotides of the invention are employed for diagnosis 35 of pathological conditions or genetic disease that involve T cell neoplasms or T cell hyperfunction or hypofunction, and more specifically conditions and diseases that involve alterations in the

structure or abundance of NF-AT<sub>c</sub> polypeptide, NF-AT<sub>c</sub> polynucleotide sequence, or structure of the NF-AT<sub>c</sub> gene or flanking region(s).

The invention also provides antibodies which bind to NF-AT<sub>c</sub> with an affinity of about at least  $1 \times 10^7 \text{ M}^{-1}$  and which lack specific high affinity binding for other proteins present in activated T cells. Such antibodies can be used as diagnostic reagents to identify T cells (e.g., activatable T cells) in a cellular sample from a patient (e.g., a lymphocyte sample, a solid tissue biopsy) as being cells which contain an increased amount of NF-AT<sub>c</sub> protein determined by standardization of the assay to be diagnostic for activated T cells. Frequently, anti-NF-AT<sub>c</sub> antibodies are included as diagnostic reagents for immunohistopathology staining of cellular samples in situ. Additionally, anti-NF-AT<sub>c</sub> antibodies may be used therapeutically by targeted delivery to T cells (e.g., by cationization or by liposome/immunoliposome delivery).

The invention also provides NF-AT<sub>c</sub> polynucleotide probes for diagnosis of neoplasia or immune status by detection of NF-AT<sub>c</sub> mRNA in cells explanted from a patient, or detection of a pathognomonic NF-AT<sub>c</sub> allele (e.g., by RFLP or allele-specific PCR analysis). A pathognomonic NF-AT<sub>c</sub> allele is an allele which is statistically correlated with the presence of a predetermined disease or propensity to develop a disease. Typically, the detection will be by in situ hybridization using a labeled (e.g., <sup>32</sup>P, <sup>35</sup>S, <sup>14</sup>C, <sup>3</sup>H, fluorescent, biotinylated, digoxigeninylated) NF-AT<sub>c</sub> polynucleotide, although Northern blotting, dot blotting, or solution hybridization on bulk RNA or poly A<sup>+</sup> RNA isolated from a cell sample may be used, as may PCR amplification using NF-AT<sub>c</sub>-specific primers. Cells which contain an increased amount of NF-AT<sub>c</sub> mRNA as compared to standard control values for cells or cell types other than activated T cells or activatable T cells will be thereby identified as activated T cells or activatable T cells. Similarly, the detection of pathognomonic rearrangements or amplification of the NF-AT<sub>c</sub> locus or closely linked loci in a cell sample will identify the presence of a pathological condition or a predisposition to developing a pathological condition (e.g., cancer, genetic disease).

The present invention also provides a method for diagnosing T cell hypofunction of hyperfunction in a human patient, wherein a diagnostic assay (e.g., immunohistochemical staining of fixed lymphocytic cells by an antibody that specifically binds human NF-AT<sub>c</sub>) is used to determine if a predetermined pathognomonic concentration of NF-AT<sub>c</sub> protein or NF-AT<sub>c</sub> mRNA is present in a biological sample from a human patient; if the assay indicates the presence of NF-AT<sub>c</sub> protein or NF-AT<sub>c</sub> mRNA at or above such predetermined pathognomonic concentration, the patient is diagnosed as having T cell hyperfunction or hypofunction condition, or transplant rejection and the like. Alternatively, T cell hypofunction or immunosuppression can be diagnosed by determining the level of nuclear and/or cytoplasmic NF-AT in a subject and comparing the level with that of a normal subject. In one embodiment, the level of nuclear and/or cytoplasmic NF-AT is determined after incubation of lymphocytes of a subject with a T cell

activator. A lower level of nuclear NF-AT relative to the normal subject indicates that the subject is immunosuppressed. A similar method can be used to monitor the state of immunosuppression in a subject who is being treated with an immunosuppressive drug, e.g., cyclosporin A. This allows more optimal dosages of the immunosuppressive drug to be administered to the subject.

5 All publications and patent applications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

#### Brief Description of the Drawings

10 Figure 1A-1C shows the nucleotide sequence of the human NF-AT<sub>c</sub> cDNA (SEQ ID NO: 45) and the deduced amino acid sequence (SEQ ID NO: 46). N indicates that a sequence ambiguity is present.

Figure 2 shows the expression of NF-AT<sub>c</sub> protein in T cells (Jurkat) and non-T cells (Cos).

15 Figure 3A and 3B show that the NF-AT<sub>c</sub> cDNA clone encodes a protein that activates transcription from an NF-AT site and is capable of activating the IL-2 promoter in non-T cells.

Figure 4 shows homology in the Rel homology domain between NF-AT<sub>c</sub>, NF-AT<sub>p</sub>, and Rel family members. The protein sequences of murine NF-AT<sub>p</sub> and the Rel proteins Dorsal (the Drosophila axis-determining protein) (SEQ ID NO: 47), human c-Rel (SEQ ID NO: 48), NF- $\kappa$ B p50 (SEQ ID NO: 49), and NF- $\kappa$ B p65 (SEQ ID NO: 50) are aligned to the sequence of NF-AT<sub>c</sub> (SEQ ID NO: 51) and NF-AT<sub>p</sub> (SEQ ID NO: 52). Numbering is with respect to NF-AT<sub>c</sub>. Identity to NF-AT<sub>c</sub>, open boxes; similarity in known residue function or structure, shaded areas. Stars indicate regions in which NF-AT<sub>c</sub> has: 1) a charge reversal relative to the majority of other Rel proteins, or has 2) replaced a potential salt bridge residue with a histidine or other chelating residue. Lower portion shows a schematic of NF-AT<sub>c</sub> and NF-AT<sub>p</sub>.

25 Figure 5, panel A, shows ribonuclease protection for human NF-AT<sub>c</sub> with RNA from Jurkat cells (lanes 1-6) or Hela cells (lane 7). The expected specific ribonuclease-resistant fragment is 304 nucleotides (arrow). Hela cells were non-stimulated. Jurkat cells were either non-stimulated or stimulated with 20ng/ml PMA and 2uM ionomycin for 3 hours, plus or minus 100ng/ml CsA added at the indicated times after stimulation.

30 Figure 5, panel B, shows RNA from the following human cells: KJ (preB cell ALL), JD-1 (B cell lineage ALL), K562 (erythroleukemia cell line), CML (bone marrow cells from a patient with a myeloid leukemia), human muscle tissue, Hep G2 (liver cell line), HPB ALL (T cell line, nonstimulated or stimulated with 2ug/ml PHA and 50ng/ml PMA for 30 minutes), and Hela cells analyzed by ribonuclease protection. A longer exposure of this gel indicates that the K562 cell line contains a small amount of NF-AT<sub>c</sub> transcript.

Figure 5, panel C, shows NF-AT<sub>c</sub> (upper panel) and NF-AT (lower panel) mRNA expression in mouse tissues and a skin tumor derived from NF-AT-Tag transgenic mice (Verweij et al. (1990) *J. Biol. Chem.* 265: 15788-15795). Cells were either non-stimulated or stimulated with 20ng/ml PMA and 2uM ionomycin for 3 hours. RNA was measured by quantitative 5 ribonuclease protection using murine cDNA probes. The predicted size of the fragment homologous to the probe is indicated by the arrows.

Figure 6, panel A, shows Cos cells and Jurkat cells that were transfected with reporter constructs for NF-AT or HNF-1 ( $\beta$ 28). Co-transfected expression vectors for NF-AT<sub>c</sub> (+NF-AT) or HNF-1<sub>a</sub> (+HNF-1) were included where indicated, otherwise empty pBJ5 vector was included. 10 Cells were stimulated as indicated: PMA, P + I (PMA plus ionomycin).

Figure 6, panel B, shows Cos cells that were transfected with IL-2 luciferase and with expression vectors as in panel A. Stimulation were as in panel A. Data in panel A and panel B are expressed as fold induction of luciferase activity over nonstimulated value with empty pBJ5 vector. Bars represent mean and range of 2-3 independent transfections.

Figure 6, panel C, shows that expression of NF-AT<sub>c</sub> in Cos cells gives rise to specific 15 DNA binding activity. Gel mobility shifts using nuclear extracts from Cos cells transfected with pBJ5 (lanes 1 and 3), with NF-AT<sub>c</sub> (lanes 2 and 4-7), from non-transfected Jurkat cells (lanes 8-11) or using cytosols from pBJ5- or NF-AT<sub>c</sub>-transfected Cos cells (lanes 12-13, 15-16) combined 20 with Hela nuclear extract (lanes 15-16). Lane 14, Hela nuclear extract alone. Labeled AP-1 (lanes 1-2) or NF-AT (lanes 3-16) probes and cold competitor oligonucleotides are indicated. Arrows indicate specific AP-1 and NF-AT complexes.

Figure 6, panel D, shows antisera induced supershift of NF-AT. NF-AT and AP-1 gel 25 mobility shifts using nuclear extracts from stimulated Jurkat cells or murine thymocytes. Either no antisera, preimmune, or one of two different immune antisera was included as indicated. Arrows indicate specific NF-AT or AP1 complexes or supershifted NF-AT complexes (\*).

Figure 7 shows dominant-negative NF-AT<sub>c</sub>. Jurkat Tag cells were transfected with vector 30 plasmid (control) or with the dominant negative NF-AT<sub>c</sub> plasmid, plus the indicated secreted alkaline phosphatase reporter plasmid. Transfected cells were transferred to fresh culture medium 24 hours after transfection and secreted alkaline phosphatase activity was measured (Clipstone and Crabtree (1992) *Nature* 357: 695-698) 16 to 24 hours later, after stimulation with 1 uM ionomycin plus 20 ng/ml PMA (NF-AT and IL-2 reporters), 20 ng/ml PMA alone (API reporter) or no stimulation (RSV reporter). Bars indicate, secreted alkaline phosphatase activity from cells 35 transfected with the dominant negative NF-AT<sub>c</sub> as a percentage of the activity from cells transfected in parallel with control plasmid, and represent data obtained from (n) independent transfections. The dominant negative NF-AT<sub>c</sub> consists of a carboxy terminal truncation of the epitope tagged NF-AT<sub>c</sub> expression plasmid extending to the PvuII site at amino acid 463.

Figure 8 shows changes in mobility of epitope tagged NF-AT<sub>c</sub> expressed in Jurkat cells. Cells were transfected with NF-AT<sub>c</sub> as in Fig. 2 and stimulated as shown for 2 hrs plus or minus 100ng/ml CsA. Whole cell lysates were analyzed by western blotting as in Fig. 2.

Figure 9, panel A, is a diagram of a FLAG epitope tagged NF-ATc1 protein indicating the respective positions in SEQ ID NO: 38 of the Rel Similarity Domain (RSD), two nuclear localization sequences (NLS), a conserved domain rich in serines (SRR), and three repeats rich in serines and prolines (SP1, SP2, SP3).

Figure 9, panel B, is a diagram showing the percentage of Cos cells transiently transfected with NF-ATc1 (SH160c) expressing NF-ATc1 in the nucleus after various times of treatment of the cells with ionomycin and calcium (I + Ca<sup>++</sup>), or with FK506 plus (I+Ca<sup>++</sup> + FK506) or with ionomycin plus 2.5 mM EGTA (I + EGTA) for 60 minutes.

Figure 9, panel C, is a diagram of the NF-AT fusion protein CΔ418-GFP, containing amino acids 1-418 of NF-ATc1 including the serine rich domain (SRR) and the three serine-proline rich domain (SP1, SP2, and SP3) fused to the green fluorescent protein (GFP).

Figure 9, panel D, shows the percentage of Cos cells transiently transfected with constructs encoding NF-ATc1 (□) or NF-AT(CΔ418)-GFP (○) having cytoplasmic NF-AT after one hour of stimulation with ionomycin and calcium and then replacement of the medium with medium containing FK506. Cells expressing NF-ATc in the cytoplasm and those expressing NF-ATc in both cytoplasm and nucleus were added and divided by the total number of analyzed expressing cells.

Figure 10 depicts a diagram showing the percentage of Cos cells expressing NF-AT in the nuclear following transient transfection of the cells with a construct encoding NF-ATc1 fused to zero, one, or two copies of a wild-type NLS from SV40 large T antigen [NLS] inserted between the FLAG epitope and the second amino acid of NF-ATc1, or one or two copies of a mutant form of the NLS (NLS-T). Cells were stained with the anti-FLAG antibody.

Figure 11, panel A, shows the amino acid sequence of two conserved putative NLSs in NF-ATc1 (SEQ ID NO: 53 and SEQ ID NO: 54), and their positions in SEQ ID NO: 38.

Figure 11, panel B, shows a diagram of NF-ATc1 and the amino acid sequence of the two NLSs, above which are shown the amino acid sequence of the mutated NLSs (TRTG has SEQ ID NO: 55 and KRKK has SEQ ID NO: 56). The lower portion of the figure shows the percentage of Cos cells expressing wildtype NF-AT or mutated NF-AT (m265, corresponding to an NF-ATc1 protein having a mutated N-terminal NLS; and m265 + 682 containing the m265 mutation and a mutation of the C-terminal NLS) in the nucleus after transient transfection of the cells and stimulation with ionomycin and calcium for 60 minutes. The percentage of cells staining in nucleus (lighted shaded areas), cytoplasm (solid areas), or both compartments (darkly shaded areas) was determined.

Figure 12, panel A, shows the amino acid sequence of the SRR from NF-ATc1 (amino acid 170 to 194 of SEQ ID NO: 38, set forth in SEQ ID NO: 57), and the cellular localization of NF-ATc1 proteins (nuclear (N) or cytoplasmic (C)) having the indicated serine to alanine substitutions (SEQ ID NOs: 58-63).

5 Figure 12, panel B, is a photograph of a Western blot, showing phosphorylated GST-NF-ATc1 fusion proteins containing amino acids 196-304 of NF-ATc (WT) or with serine to alanine mutations in the three SP repeats (S→A), after having been phosphorylated by incubation with [ $\gamma$ -<sup>32</sup>P]ATP and a partially purified preparation of cellular NF-AT kinase activity (brain extract) and then incubated with phosphatases as indicated, prior to separation by electrophoresis and 10 autoradiography (top lane). The bottom lane indicates the amount of NF-AT in each sample, as visualized by Coomassie staining.

15 Figure 13, panel A, is a photograph of a Western blot showing the results of an affinity purification of extracts of COS cells that had been transfected with the empty expression vector (Vector) or a vector encoding the HA epitope-tagged amino-terminal 418 residues of NF-ATc (2-418) with glutathione-agarose beads coupled to GST or incubated with beads coupled to a GST fusion with the RSD of NF-ATc (GST-RSD). Affinity-selected proteins were detected by immunoblotting with the anti-HA 12CA5 antibody. The left part of the panel is a graphic representation of the NF-AT polypeptide that was attached to the agarose beads and the NF-AT polypeptide which was affinity purified on the beads.

20 Figure 13, panel B, is a photograph of a Western blot showing the results of an affinity purification of extracts from COS cells transfected with a construct encoding the HA epitope-tagged amino-terminal 418 residues of NF-ATc (2-418) or vector alone (Vector), with GST-RSD or a version with a mutation in the carboxy-terminal NLS (mNLS). Bound proteins were detected with the 12CA5 antibody. The left part of the panel is a graphic representation of the NF-AT 25 polypeptides used in the example.

30 Figure 13, panel C, is a photograph of a Western blot showing the results of an affinity purification of extracts from COS cells that had been transfected with the HA epitope-tagged amino-terminal 418 residues of NF-ATc (1-418 WT) or versions in which S→A mutations were present in the SRR or SP repeats with GST-RSD. The associated proteins were detected with the 7A6 antibody. The lower part of the blot shows the amount of NF-AT present in the cell extracts prior to affinity purification.

35 Figure 14 shows a model of the mechanism of NT-ATc nuclear entry. According to the model, cytoplasmic NF-ATc is phosphorylated on the SP repeats and SRR masking the activity of its two partially redundant NLSs, the sequence KRK at position 265-267, and the sequence KRKK/R at position 682-685 of NF-ATc1. Dephosphorylation in response to activation of calcineurin leads to an alteration in an intramolecular interaction and perhaps a conformational

change, exposing one or more of the NLSs to the nuclear import machinery. Once in the nucleus, termination of calcium signaling results in rapid export to the cytosol, possibly by the exposure of nuclear export sequences (NES).

Figure 15, panel A, shows the amino acid sequence of amino acids 196-304 of NF-ATc1 (SEQ ID NO: 38) set forth in SEQ ID NO: 64. Putative overlapping GSK-3 consensus sites [SPXXS(P)] (Fiol *et al.*, *J. Biol. Chem.* 269, 32187 (1994)) are overlined. The nuclear localization sequence is in bold type, and sites phosphorylated by PKA *in vitro* are boxed. The underlined serines are serines that have been substituted with alanines in some examples.

Figure 15, panel B, shows a graph depicting the level of GSK-3 activity in various fractions eluted from a P-11 column following ammonium sulfate fractionation of brain extracts. The lower part of the panel shows autoradiograms of Western blots showing the ability of the eluted fractions to phosphorylate GST-NF-AT fusion proteins containing wild-type (WT) NF-ATc1 or NF-ATc1 in which the underlined serines of pane A were mutated into alanines, or NF-ATc1 which was *in vitro* phosphorylated with PKA (WT-PKA prephosph.).

Figure 15, panel C, shows a graph depicting the amount of protein in various fractions eluted from a Mono-S column of the P-11 pool of panel B. The lower part of the panel shows autoradiograms of Western blots showing the ability of the eluted fractions to phosphorylate GST-NF-AT fusion proteins containing wild-type (WT) NF-ATc1 or NF-ATc1 in which the underlined serines of pane A were mutated into alanines, or NF-ATc1 which was *in vitro* phosphorylated with PKA (WT-PKA prephosph.). The bottom two photographs show autoradiograms of Western blots containing protein from the eluted fractions incubated with antisera specific for GSK-3 $\alpha$  and GSK-3 $\beta$ .

Figure 16, panel A, shows an autoradiogram of a Western blot of PKA-prephosphorylated NF-AT (WT-PKA prephos.) incubated with brain extracts immunodepleted with antisera to GSK-3 $\alpha$  and/or GSK-3 $\beta$  or control antibodies in an *in vitro* kinase reaction with [ $\gamma$ -<sup>32</sup>P]ATP, and the <sup>32</sup>P-labeled substrate (upper gel; the lower gel shows Coomassie staining). Figure 16, panel B, shows a photograph of an autoradiogram of a Western blot of PKA-prephosphorylated NF-AT (WT-PKA prephos.) or NF-AT (WT) incubated with brain extracts immunodepleted with antisera to GSK-3 $\alpha$  and GSK-3 $\beta$  or control antibodies (Ig) in an *in vitro* kinase reaction with [ $\gamma$ -<sup>32</sup>P]ATP.

Figure 17, panel A, shows autoradiograms of a Western blot depicting NF-AT-GST fusion proteins phosphorylated *in vitro* with the indicated purified kinases. In the rightmost lanes, the first kinase was permitted to phosphorylate the WT substrate with nonradioactive ATP to completion; then, the WT substrate beads were washed to remove the kinase and the WT beads were phosphorylated by the second kinase in the presence of [ $\gamma$ -<sup>32</sup>P]ATP.

Figure 17, panel B, depicts autoradiograms of two-dimensional tryptic phosphopeptide maps of the NF-ATc1 wild-type fusion protein with the indicated kinases *in vitro*. NF-ATc was overexpressed in COS cells [which support reversible Ca<sup>2+</sup>-dependent nuclear localization] and labeled with [<sup>32</sup>P]orthophosphate. In the lower right panel, the PKA + GSK-3 $\beta$  *in vitro* phosphorylated peptides were mixed with the *in vivo* phosphorylated peptides before two-dimensional separation to establish that they are similar. Phosphopeptides migrating differently are circled with a dashed line.

Figure 18, panel A, shows the activity of alkaline phosphatase (SEAP) in extracts from Jurkat cells transfected with an SEAP reporter gene under the control of an NF-AT, AP-1 or HIV-LTR regulatory element and in which GSK-3 $\beta$  is overexpressed or in which the empty vector was overexpressed. NF-AT SEAP activity is expressed as a percentage of the ionomycin-stimulated and phorbol 12-myristate 13-acetate (PMA)-stimulated control activity; AP-1 and HIV-LTR SEAP activities are expressed as a percentage of PMA-stimulated activity (Spencer et al., *Science* 262, 1019 (1993)).

Figure 18, panel B, shows the percentage of cells expressing NF-ATc1 in the cytoplasm, nucleus or both after cotransfection of COS cells with FLAG epitope-tagged NF-ATc1 and each of the indicated serine-threonine kinases. Cells were stimulated with ionomycin and 10 mM Ca<sup>2+</sup>, and the percentages of cells expressing NF-AT localized in the nucleus, cytoplasm, or both compartments were scored visually and are presented as a percentage of expressing cells. The transfected ERK kinase was activated by adding PMA (25 ng/ml).

Figure 19 shows the percentage of cells having cytoplasmic NF-ATc, after overexpression of GSK-3 $\beta$  in COS cells cotransfected with expression constructs encoding FLAG epitope-tagged NF-ATc1, calcineurin A and B, and vector (□), GSK-3 $\beta$  (◊) or GSK-KM (○), a catalytically inactive GSK-3 $\beta$  (He et al., *Nature* 374, 617 (1995)). Cells were also cotransfected with a version of NF-ATc1 in which the underlined serines in Fig. 1A were changed to alanines with calcineurin and GSK-3 $\beta$  ( $\Delta$ ). To obtain NF-ATc localization in the nucleus, the transfected cells were treated with ionomycin and Ca<sup>2+</sup> for 60 min, then the medium was changed to medium with FK506 (20 ng/ml) to terminate Ca<sup>2+</sup> signaling and to block nuclear reentry of NF-ATc. Transfected NF-ATc was detected with FLAG mAb M2 by indirect immunofluorescence, and 200 expressing cells were scored as expressing NF-ATc in the cytoplasm, nucleus, or both compartments.

Figure 20A shows a schematic of the endogenous NF-ATc4 gene, the targeting construct used to transfect ES cells and the targeted NF-ATc4 locus resulting from homologous recombination.

Figure 20B shows a Southern blot of mouse tail DNA digested with EcoRV and blotted with the 3' outside probe

Detailed Description of the Invention*(i) Overview*

On a cellular level, the heart functions as a syncytium of myocytes and surrounding support cells, called non-myocytes. While non-myocytes are primarily fibroblast/mesenchymal cells, they 5 also include endothelial and smooth muscle cells. Indeed, although myocytes make up most of the adult myocardial mass, they represent only about 30% of the total cell numbers present in heart. Because of their close relationship with cardiac myocytes *in vivo*, non-myocytes are capable of influencing myocyte growth and/or development. This interaction may be mediated directly through cell-cell contact or indirectly via production of a paracrine factor. Such association *in vivo* 10 is important since both non-myocyte numbers and the extracellular matrix with which they interact are increased in myocardial hypertrophy and in response to injury and infarction. These changes are associated with abnormal myocardial function.

Cardiac myocytes are unable to divide shortly after birth. Further growth occurs through hypertrophy of the individual cells. Cell culture models of myocyte hypertrophy have been 15 developed to understand better the mechanisms for cardiac myocyte hypertrophy. Simpson et al., *Circ. Res.*, 51: 787-801 (1982); Chien et al., *FASEB J.*, 5: 3037-3046 (1991). Most studies of heart myocytes in culture are designed to minimize contamination by non-myocytes. See, for example, Simpson and Savion, *Cir. Cres.*, 50: 101-116 (1982); Libby, *J. Mol. Cell. Cardiol.*, 16: 803-811 (1984); Iwaki et al., *J. Biol. Chem.*, 265:13809-13817 (1990).

20 Hypertrophy of adult cardiac ventricular myocytes is a response to a variety of conditions which lead to chronic overload. This response is characterized by an increase in myocyte cell size and contractile protein content without concomitant cell division, and activation of embryonic genes, including the gene for atrial natriuretic peptide (ANP). Chien et al., *supra*. Adult myocyte hypertrophy is initially beneficial as a short term response to impaired cardiac function by 25 permitting a decrease in the load on individual muscle fibers. With severe, long-standing overload, however, the hypertrophied cells begin to deteriorate and die. Katz, "Heart failure," in Katz AM, ed., *Physiology of the Heart* (New York: Raven Press; 1992) pp. 638-668. Endothelial cells, smooth muscle cells and fibroblast/mesenchymal cells exist in close contact with myocytes in the heart. Nag, *Cytobios.*, 28: 41-61 (1980).

30 One aspect of the present invention relates to the use of agents to inhibit NF-AT activity, particularly NF-AT<sub>c3</sub> or NF-AT<sub>c4</sub>, as part of a method for inhibiting or preventing unwanted growth of cardiac and other vascular tissues. In considering the potential of any protein as a target for drug development, one must consider if the loss-of-function of the protein would be, over the therapeutic dosage, lethal to the treated subject, e.g., either because of systemic lethality or 35 lethality to the treated tissue. In the art, transgenic animals having loss-of-function mutations to NF-AT alleles have indicated that inhibition of certain NF-AT functions includes a lethal

consequence. Likewise, loss-of-function of calcineurin can also result in a lethal phenotype. However, as described herein (see, e.g., Example 20), neither loss-of-function of NF-ATc4 or NF-ATc3 is lethal to the animal, nor do such mutations have any untoward effects on the pathology of normal cardiac tissue. Our observations suggest, therefore, that NF-ATc4 or NF-ATc3 are 5 appropriate targets for drug development.

Thus, according to the present invention, there is provided a method for the inhibiting the growth of cardiac and vascular tissue, e.g., which inhibits growth of myocytic and/or non-myocytic cells in such tissue. For example, as described below, the subject method can be used to slow the process of cardiac hypertrophy and arteriolar smooth muscle proliferation, e.g., as part 10 of a treatment of vascular smooth muscle hypertrophy or cardiac hypertrophy. Chronic cardiac hypertrophy, for example, is a significantly diseased state which is a precursor to congestive heart failure and cardiac arrest. Antagonists of NF-AT, and particularly of NF-AT<sub>c3</sub> or NF-AT<sub>c4</sub>, are useful as part of a treatments for congestive heart failure.

Moreover, based on differences in specificity for DNA recognition elements, and in 15 the protein-protein interactions that the various NF-AT paralogs have, the present invention specifically contemplates the identification, and use, of NF-AT antagonists which selectively inhibit the activity of certain of the NF-ATproteins. In preferred embodiments, the present invention contemplates a method for treating cardiac hypertrophy, or other preventing other growth of cardiac and vascular tissue, through the use of NF-AT antagonists which are selective 20 for NF-AT<sub>c3</sub> and/or NF-AT<sub>c4</sub>, but not NF-AT<sub>c1</sub> or NF-AT<sub>c2</sub>. For instance, the NF-AT antagonist can be selected so as to have an ED50 for inhibition of NF-AT<sub>c3</sub> or NF-AT<sub>c4</sub> in vivo of at least one, and more preferably, two, three, four and even five orders of magnitude less than its ED50 for inhibition of NF-AT<sub>c1</sub> or NF-AT<sub>c2</sub> activity.

In other embodiments, antagonists of NF-AT can be used to inhibit growth of cancers 25 and other hyperproliferative disorders involving tissue of muscle origin, such as in the treatment of rhabdomyosarcoma and leiomyoma.

#### *(ii) Definitions*

Unless defined otherwise, all technical and scientific terms used herein have the same 30 meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

Generally, the nomenclature used hereafter and the laboratory procedures in cell culture, 35 molecular genetics, and nucleic acid chemistry and hybridization described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic

acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection). Generally enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see, 5 generally, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference) which are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

10 Oligonucleotides can be synthesized on an Applied Bio Systems oligonucleotide synthesizer according to specifications provided by the manufacturer.

Methods for PCR amplification are described in the art (PCR Technology: Principles and Applications for DNA Amplification ed. H.A. Erlich, Freeman Press, New York, NY (1992); PCR Protocols: A Guide to Methods and Applications, eds. Innis, Gelfland, Sninsky, and White, 15 Academic Press, San Diego, CA (1990); Mattila et al. (1991) Nucleic Acids Res. 19: 4967; Eckert, K.A. and Kunkel, T.A. (1991) PCR Methods and Applications 1: 17; PCR, eds. McPherson, Quirk, and Taylor, IRL Press, Oxford; and U.S. Patent 4,683,202, which are incorporated herein by reference).

20 As used herein, the terms "exemplary NF-AT nucleic acid" and "exemplary NF-AT protein" refer to the nucleotide and amino acid sequences, respectively, of such NF-AT genes as described in the examples below, as well as in such references as Northrop et al. (1994) Nature 369:497; Park et al. (1996) J. Biol. Chem. 271:20914; Luo et al. (1996) MCB 16:3955; Hoey et al. (1995) Immunity 2:461; Masuda et al. (1995) Mol. Cell. Biol. 15:2697; and US Patent Nos. 5,708,158 and 5,612,455. These references, among others, provide the sequences of human NF-25 AT3 (i.e., human NF-ATc4), and those of three splice variants of human NF-AT4 (i.e., human NF-ATc3). The three forms of NF-AT4 have been designated NF-AT4a, NF-AT4b, and NF-AT4c, and the positions of the splice junctions in the coding regions are after proline 699 in NF-AT4a, and after valine 700 and proline 716 in NF-AT4b and NF-AT4c. An alignment of the Rel domains of these NF-AT proteins shows that certain areas are particularly well conserved. In particular, 30 the following amino acid sequences are found in the REL domain of all NF-AT polypeptides: the amino acid sequence HHRAHYETEGSRGAVKA (SEQ ID NO: ), the amino acid sequence PHAFYQVHRITGK (SEQ ID NO: ), the amino acid sequence DIELRKGETDGRKNTRVRLVFRVHX1P (SEQ ID NO: ), and the amino acid sequence PX2ECSQRSAX3ELP (SEQ ID NO: ), wherein each X1 and X2 is hydrophobic residue such as 35 valine or isoleucine, and X3 is any residue, but preferably glutamine or histidine.

The GenBank Accession Numbers of the exemplary human NF-AT nucleic acids and polypeptides are provided in the following Table:

|    |                |                                      |
|----|----------------|--------------------------------------|
|    | <u>NF-AT</u>   | <u>GenBank No.</u>                   |
| 5  | <u>NF-ATc1</u> |                                      |
|    | NF-ATc         | U08015                               |
|    | NF-ATc.b       | U59736                               |
|    | <u>NF-ATc2</u> |                                      |
| 10 | NF-AT1         | I38152                               |
|    | NF-ATp1        | U43341 isoform B<br>U43342 isoform C |
|    | <u>NF-ATc3</u> |                                      |
| 15 | NF-AT4a        | I38155                               |
|    | NF-AT4b        | I38156                               |
|    | NF-AT4c        | L41067                               |
|    | <u>NF-ATc4</u> |                                      |
| 20 | NF-AT3         | L41066<br>I38154                     |
|    | NF-ATx         | U14510                               |
|    | NF-ATx2        | U85428                               |
|    | NF-ATx3        | U85429                               |
| 25 | NF-ATx4        | U85430                               |

NF-ATc2 has also been referred to as NFIL2E and NFII-a.

Other examples of NF-AT genes and genes products can be found in GenBank, particularly accessions I80836, U36576, U36575, I60722, U02079, AF049606, AF087434, as well as PRF locus 2013343A, PIR locus S45262 and A48753.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) hypertrophy. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in which the disorder is to be prevented. The hypertrophy may be from any cause, including congenital, viral, idiopathic, cardiotoxic, or myotoxic causes, or as a result of ischemia or ischemic insults such as myocardial infarction. Typically, the treatment is performed to stop or slow the progression of hypertrophy, especially after heart damage, such as from ischemia, has occurred. Preferably, for treatment of myocardial infarctions, the agent(s) is given immediately after the myocardial infarction, to prevent or lessen hypertrophy.

40 By the term "heart failure" is meant an abnormality of cardiac function where the heart does not pump blood at the rate needed for the requirements of metabolizing tissues. Heart failure includes a wide range of disease states such as congestive heart failure, myocardial infarction, tachyarrhythmia, familial hypertrophic cardiomyopathy, ischemic heart disease, idiopathic dilated cardiomyopathy, and myocarditis. The heart failure can be caused by any number of factors,

including ischemic, congenital, rheumatic, or idiopathic forms. Chronic cardiac hypertrophy is a significantly diseased state which is a precursor to congestive heart failure and cardiac arrest.

Still more specifically, the terms "treating" and "treatment" shall mean preventing, alleviating, and/or inhibiting. In this regard, the method of the present invention can be used as 5 part of a treatment for, but not limited to, (1) ventricular muscle cell hypertrophy, e.g., which is induced by  $\alpha$ 1-adrenergic agonists and/or endothelin, (2) ventricular muscle cell hypertrophy induced by drugs which have an adverse side effect of promoting cardiac hypertrophy, (3) a medical condition, e.g., heart failure, mediated by ventricular muscle cell hypertrophy, and (4) ventricular muscle cell hypertrophy initiated by cardiac injury, such as viral myocarditis, long-standing hypertension, cardiomyopathy due to pathological stimuli, and post-myocardial 10 infarction.

"Chronic" administration refers to administration of the agent(s) in a continuous mode as opposed to an acute mode, so as to maintain the initial anti-hypertrophic effect for an extended period of time.

15 The term "NF-AT antagonist" as used herein refers to any molecule which blocks or prevents the NF-AT dependent transcription. Such antagonists accomplish this effect in various ways. For instance, one class of antagonists will bind to an NF-AT protein with sufficient affinity and specificity to inhibit NF-AT interaction with its cognate response element in DNA or protein factors, such as AP1. Other classes of antagonist will bind to NF-AT and inhibit its nuclear 20 localization, such as by preventing phosphorylation of NLS sites, or inhibiting conformation changes resulting from phosphorylation. Still another class of antagonists can prevent NF-AT activity by inhibiting kinases, such as GSK-3 or PKA, from phosphorylating NLS sites on the NF-AT protein. Other antagonists are described herein and will be apparent to those skilled in the art.

25 "Ventricular muscle cell hypertrophy" is a condition characterized by an increase in the size of individual ventricular muscle cells, the increase in cell size being sufficient to result in a clinical diagnosis of the patient or sufficient as to allow the cells to be determined as larger (e.g., 2-fold or more larger than non-hypertrophic cells). It may be accompanied by accumulation of contractile proteins within the individual cardiac cells and activation of embryonic gene expression.

30 "Suppression" of ventricular muscle cell hypertrophy means a reduction in one of the parameters indicating hypertrophy relative to the hypertrophic condition, or a prevention of an increase in one of the parameters indicating hypertrophy relative to the normal condition. For example, suppression of ventricular muscle cell hypertrophy can be measured as a reduction in cell size relative to the hypertrophic condition. Suppression of ventricular muscle cell hypertrophy means a decrease of cell size of 10% or greater relative to that observed in the hypertrophic condition. More preferably, suppression of hypertrophy means a decrease of cell size of 30% or 35

greater; most preferably, suppression of hypertrophy means a decrease of cell size of 50% or more. Relative to the hypertrophy score assay when phenylephrine is used as the inducing agent, these decreases would correlate with hypertrophy scores of about 6.5 or less, 5.0-5.5, and 4.0-5.0, respectively. When a different agent is used as the inducing agent, suppression is measured 5 relative to the maximum cell size (or hypertrophic score) measured in the presence of that inducer.

"Prevention of ventricular muscle cell hypertrophy" is determined by preventing an increase in cell size relative to normal cells, in the presence of a concentration of inducer sufficient to fully induce hypertrophy. For example, prevention of hypertrophy means a cell size increase less than 200% greater than non-induced cells in the presence of a maximally-stimulating 10 concentration of inducer. More preferably, prevention of hypertrophy means a cell size increase less than 135% greater than non-induced cells; and most preferably, prevention means a cell size increase less than 90% greater than non-induced cells. Relative to the hypertrophy score assay when phenylcphrine is used as the inducing agent, prevention of hypertrophy in the presence of a maximally-stimulating concentration of phenylephrine means a hypertrophic score of about 15 6.0-6.5, 5.0-5.5, and 4.0-4.5, respectively.

By the term "effective amount" or "therapeutically effective amount" of an NF-AT therapeutic, e.g., antagonist is meant an amount of an NF-AT therapeutic sufficient to obtain the desired physiological effect, e.g., suppression of ventricular muscle cell hypertrophy. An effective amount of an NF-AT therapeutic is determined by the care giver in each case on the basis 20 of factors normally considered by one skilled in the art to determine appropriate dosages, including the age, sex, and weight of the subject to be treated, the condition being treated, and the severity of the medical condition being treated.

As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage (Immunology - A Synthesis, 2nd Edition, E.S. Golub and D.R. Gren, Eds., 25 Sinauer Associates, Sunderland, Massachusetts (1991), which is incorporated herein by reference). Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as  $\alpha,\alpha$ -disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples 30 of unconventional amino acids include: 4-hydroxyproline,  $\gamma$ -carboxyglutamate,  $\epsilon$ -N,N,N-trimethyllysine,  $\epsilon$ -N-acetyllysine, O-phosphoserine, N-acetylsersine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine,  $\omega$ -N-methylarginine, and other similar amino acids and imino 35 acids (e.g., 4-hydroxyproline). In the polypeptide notation used herein, the lefthand direction is the amino terminal direction and the righthand direction is the carboxy-terminal direction, in accordance with standard usage and convention. Similarly, unless specified otherwise, the lefthand end of single-stranded polynucleotide sequences is the 5' end; the lefthand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to

3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA and which are 5' to the 5' end of the RNA transcript are referred to as "upstream sequences"; sequence regions on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the RNA transcript are 5 referred to as "downstream sequences".

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

10 The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence.  
15 For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence 20 used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, such as a polynucleotide sequence of Fig. 1, or may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides 25 may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A 30 "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal 35 alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv.

Appl. Math. 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48: 443, by the search for similarity method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (c.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence, for example, as a segment of the full-length human NF-AT<sub>c</sub> polynucleotide sequence shown in Fig. 1 or the full-length murine or bovine NF-AT<sub>c</sub> cDNA sequence.

As applied to polypeptides, a degree of identity of amino acid sequences is a function of the number of identical amino acids at positions shared by the amino acid sequences. A degree of homology or similarity of amino acid sequences is a function of the number of amino acids, i.e. structurally related, at positions shared by the amino acid sequences. An "unrelated" or "non-homologous" sequence shares less than 40 % identity, though preferably less than 25 % identity, with one of the NF-AT<sub>c</sub> sequences of the present invention. The term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side

chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, 5 arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The term "NF-AT<sub>c</sub> native protein" and "full-length NF-AT<sub>c</sub> protein" as used herein refers to a naturally-occurring NF-AT<sub>c</sub> polypeptide corresponding to the deduced amino acid sequence 10 shown in Fig. 1 or corresponding to the deduced amino acid sequence of a cognate full-length cDNA. Also for example, a native NF-AT<sub>c</sub> protein present in naturally-occurring lymphocytes which express the NF-AT<sub>c</sub> gene are considered full-length NF-AT<sub>c</sub> proteins.

The term "NF-AT<sub>c</sub> fragment" as used herein refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion, but where the remaining amino acid sequence is 15 identical to the corresponding positions in the NF-AT<sub>c</sub> sequence deduced from a full-length cDNA sequence (e.g., the cDNA sequence shown in Fig. 1). NF-AT<sub>c</sub> fragments typically are at least 14 amino acids long, preferably at least 20 amino acids long, usually at least 50 amino acids long or longer.

The term "NF-AT<sub>c</sub> analog" as used herein refers to polypeptides which are comprised of 20 a segment of at least 25 amino acids that has substantial identity to a portion of the deduced amino acid sequence shown in Fig. 1, and which has at least one of the following properties: (1) binding to other polypeptides under suitable binding conditions including (a) other NF-AT proteins (e.g., AP-1); (b) a kinase, such as GSK-3 and PKA; (c) a phosphatase, such as calcineurin; (d) NF-AT polypeptides, in particular portions thereof, such as an NLS, SRR, SP1, SP2, and/or SP3; (2) 25 binding to a nucleic acid; (3) ability to localize to the nucleus upon T cell activation; and (4) the ability to translocate from the nucleus to the cytoplasm after termination of the stimulatory signal. Typically, NF-AT<sub>c</sub> analog polypeptides comprise a conservative amino acid substitution (or addition or deletion) with respect to the naturally-occurring sequence. NF-AT<sub>c</sub> analogs typically are at least 20 amino acids long, preferably at least 50 amino acids long or longer, most usually 30 being as long as full-length naturally-occurring NF-AT<sub>c</sub> (e.g., as shown in Fig. 1). Some NF-AT<sub>c</sub> analogs may lack biological activity but may still be employed for various uses, such as for raising antibodies to NF-AT<sub>c</sub> epitopes, as an immunological reagent to detect and/or purify  $\alpha$ -NF-AT<sub>c</sub> antibodies by affinity chromatography, or as a competitive or noncompetitive agonist, antagonist, or partial agonist of native NF-AT<sub>c</sub> protein function.

35 The term "NF-AT<sub>c</sub> polypeptide" which is used herein interchangeably with the term "NF-AT" polypeptide, is used herein as a generic term to refer to native protein, fragments, or analogs

of NF-AT<sub>c</sub>. Hence, native NF-AT<sub>c</sub>, fragments of NF-AT<sub>c</sub>, and analogs of NF-AT<sub>c</sub> are species of the NF-AT<sub>c</sub> polypeptide genus. Preferred NF-AT<sub>c</sub> polypeptides include: the human full-length NF-AT<sub>c</sub> protein comprising the polypeptide sequence shown in Fig. 1 (which is also referred to as "NF-ATc1"), or polypeptides consisting essentially of a sequence shown in Table II. Thus, the 5 genus NF-ATc includes all NF-AT polypeptides identified so far as well as those that have not yet been identified and which could be identified, e.g., by low stringency hybridization. In addition to the NF-ATc having SEQ ID NO: 38, which is also referred to as NF-ATc1, and homologs of other species, the NF-ATc genus includes NF-ATc2 (also termed NF-ATp), NF-ATc3 (also termed NF-AT4 or NF-ATx), NF-ATc4 (also termed NF-AT3), and splice variants thereof.

10 The term "cognate" as used herein refers to a gene sequence that is evolutionarily and functionally related between species. For example but not limitation, in the human genome, the human CD4 gene is the cognate gene to the mouse CD4 gene, since the sequences and structures of these two genes indicate that they are highly homologous and both genes encode a protein which functions in signaling T cell activation through MHC class II-restricted antigen recognition. 15 Thus, the cognate murine gene to the human NF-AT<sub>c</sub> gene is the murine gene which encodes an expressed protein which has the greatest degree of sequence identity to the human NF-AT<sub>c</sub> protein and which exhibits an expression pattern similar to that of the human NF-AT<sub>c</sub> (e.g., expressed in T lineage cells). Preferred cognate NF-AT<sub>c</sub> genes are: rat NF-AT<sub>c</sub>, rabbit NF-AT<sub>c</sub>, canine NF-AT<sub>c</sub>, nonhuman primate NF-AT<sub>c</sub>, porcine NF-AT<sub>c</sub>, bovine NF-AT<sub>c</sub>, and hamster NF-AT<sub>c</sub>.

20 The term "NF-AT<sub>c</sub>-dependent gene" is used herein to refer to genes which: (1) have a NF-AT binding site (a site which can be specifically footprinted by NF-AT under suitable binding conditions) within about 10 kilobases of the first coding sequence of said gene, and (2) manifest an altered rate of transcription, either increased or decreased, from a major or minor transcriptional start site for said gene, wherein such alteration in transcriptional rate correlates with the presence 25 of NF-AT<sub>c</sub> polypeptide in NF-AT complexes, such as in an activated T cell.

30 The term "altered ability to modulate" is used herein to refer to the capacity to either enhance or inhibit a biological activity, e.g., transcription of a gene; such enhancement or inhibition may be contingent on the occurrence of a specific event, such as T cell stimulation. For example, this alteration may be manifest as an inhibition of the transcriptional enhancement of the IL-2 gene that normally ensues following T cell stimulation. The altered ability to modulate transcriptional enhancement or inhibition may affect the inducible transcription of a gene, such as in the just-cited IL-2 example, or may effect the basal level transcription of a gene, or both.

35 The term "agent" is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Agents are evaluated

for potential activity as immunomodulatory agents (e.g., immunosuppressants) by inclusion in screening assays described hereinbelow.

The term "candidate immunomodulatory agent" is used herein to refer to an agent which is identified by one or more screening method(s) of the invention as a putative immunomodulatory agent. Some candidate immunomodulatory agents may have therapeutic potential as drugs for human use.

As used herein, the terms "label" or "labeled" refers to incorporation of a detectable marker, *e.g.*, by incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (*e.g.*, streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or colorimetric methods). Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes (*e.g.*,  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{35}\text{S}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ), fluorescent labels (*e.g.*, FITC, rhodamine, lanthanide phosphors), enzymatic labels (*e.g.*, horseradish peroxidase,  $\beta$ -galactosidase, luciferase, alkaline phosphatase), biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (*e.g.*, leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

As used herein, "substantially pure" means an object species is the predominant species present (*i.e.*, on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

As used herein the terms "pathognomonic concentration", "pathognomonic amount", and "pathognomonic staining pattern" refer to a concentration, amount, or localization pattern, respectively, of a NF-AT<sub>c</sub> protein or mRNA in a sample, that indicates the presence of a hypofunctional or hyperfunctional T cell condition or a predisposition to developing a disease, such as graft rejection. A pathognomonic amount is an amount of a NF-AT<sub>c</sub> protein or NF-AT<sub>c</sub> mRNA in a cell or cellular sample that falls outside the range of normal clinical values that is established by prospective and/or retrospective statistical clinical studies. Generally, an individual having a neoplastic disease (*e.g.*, lymphocytic leukemia) or T cell-mediated immune response will exhibit an amount of NF-AT<sub>c</sub> protein or mRNA in a cell or tissue sample that is higher than the

range of concentrations that characterize normal, undiseased individuals; typically the pathognomonic concentration is at least about one standard deviation above the mean normal value, more usually it is at least about two standard deviations or more above the mean normal value. However, essentially all clinical diagnostic tests produce some percentage of false positives  
5 and false negatives. The sensitivity and selectivity of the diagnostic assay must be sufficient to satisfy the diagnostic objective and any relevant regulatory requirements. In general, the diagnostic methods of the invention are used to identify individuals as disease candidates, providing an additional parameter in a differential diagnosis of disease made by a competent health professional.

10

*(iii) Exemplary Embodiments*

A. NF-AT<sub>c</sub> Polynucleotides

Genomic or cDNA clones encoding NF-AT<sub>c</sub> may be isolated from clone libraries (e.g., available from Clontech, Palo Alto, CA) using hybridization probes designed on the basis of the  
15 nucleotide sequences shown in Fig. 1, or other exemplary NF-AT sequences, and using conventional hybridization screening methods (e.g., Benton WD and Davis RW (1977) Science 196: 180; Goodspeed et al. (1989) Gene 76: 1; Dunn et al. (1989) J. Biol. Chem. 264: 13057). Where a cDNA clone is desired, clone libraries containing cDNA derived from human mRNA is preferred. Alternatively, synthetic polynucleotide sequences corresponding to all or part of the  
20 sequences shown in Fig. 1, or other exemplary NF-AT sequences, may be constructed by chemical synthesis of oligonucleotides. Additionally, polymerase chain reaction (PCR) using primers based on the sequence of an NF-AT gene may be used to amplify DNA fragments from genomic DNA, mRNA pools, or from cDNA clone libraries. U.S. Patents 4,683,195 and 4,683,202 describe the PCR method. Additionally, PCR methods employing one primer that is based on the  
25 sequence data disclosed in, e.g., Fig. 1 and a second primer that is not based on that sequence data may be used. For example, a second primer that is homologous to or complementary to a polyadenylation segment may be used. In an embodiment, a polynucleotide comprising the 2742 nucleotide long sequence of Fig. 1 can also be readily constructed by those of skill in the art by using the degeneracy of the genetic code. Polynucleotides encoding amino acids 418 to 710 of the  
30 NF-AT<sub>c</sub> sequence of Fig. 1 can also be constructed by those of skill in the art.

It is apparent to one of skill in the art that nucleotide substitutions, deletions, and additions may be incorporated into the polynucleotides of the invention. Nucleotide sequence variation may result from sequence polymorphisms of various NF-AT<sub>c</sub> alleles, minor sequencing errors, and the like. However, such nucleotide substitutions, deletions, and additions should not substantially  
35 disrupt the ability of the polynucleotide to hybridize to one of the polynucleotide sequences shown

in Fig. 1 under hybridization conditions that are sufficiently stringent to result in specific hybridization.

Specific hybridization is defined herein as the formation of hybrids between a probe polynucleotide (e.g., a polynucleotide of the invention which may include substitutions, deletion, 5 and/or additions) and a specific target polynucleotide (e.g., a polynucleotide having the sequence in Fig. 1, or other exemplary NF-AT sequences), wherein the probe preferentially hybridizes to the specific target such that, for example, a single band corresponding to NF-AT<sub>c</sub> mRNA (or bands corresponding to multiple alternative splicing products of the NF-AT<sub>c</sub> gene) can be identified on a Northern blot of RNA prepared from a suitable cell source (e.g., a T cell expressing NF-AT<sub>c</sub>).  
10 Polynucleotides of the invention and recombinantly produced NF-AT<sub>c</sub>, and fragments or analogs thereof, may be prepared on the basis of the sequence data provided in Fig. 1, or other exemplary NF-AT sequences, according to methods known in the art and described in Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., (1989), Cold Spring Harbor, N.Y. and Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques 15 (1987), Academic Press, Inc., San Diego, CA, which are incorporated herein by reference.

NF-AT<sub>c</sub> polynucleotides may be short oligonucleotides (e.g., 25-100 bases long), such as for use as hybridization probes and PCR (or LCR) primers. NF-AT<sub>c</sub> polynucleotide sequences may also comprise part of a larger polynucleotide (e.g., a cloning vector comprising a NF-AT<sub>c</sub> clone) and may be fused, by polynucleotide linkage, in frame with another polynucleotide 20 sequence encoding a different protein (e.g., glutathione S-transferase or  $\beta$ -galactosidase) for encoding expression of a fusion protein. Typically, NF-AT<sub>c</sub> polynucleotides comprise at least 25 consecutive nucleotides which are substantially identical to a naturally-occurring NF-AT<sub>c</sub> sequence (e.g., Fig. 1, or other exemplary NF-AT sequences), more usually NF-AT<sub>c</sub> 25 polynucleotides comprise at least 50 to 100 consecutive nucleotides which are substantially identical to a naturally-occurring NF-AT<sub>c</sub> sequence. However, it will be recognized by those of skill that the minimum length of a NF-AT<sub>c</sub> polynucleotide required for specific hybridization to a NF-AT<sub>c</sub> target sequence will depend on several factors: G/C content, positioning of mismatched bases (if any), degree of uniqueness of the sequence as compared to the population of target 30 polynucleotides, and chemical nature of the polynucleotide (e.g., methylphosphonate backbone, phosphorothiolate, etc.), among others.

For example but not limitation, suitable hybridization probes for detecting and/or quantifying the presence of NF-AT<sub>c</sub> mRNA in a sample generally comprise at least one, preferably at least two, and more preferably all of the following human NF-AT<sub>c</sub> sequences shown in Table I, or their complements:

5'-TTC CTC CGG GGC GCG CGG CGT GAG CCC GGG GCG AGG-3' (SEQ ID NO: 1);  
 5'-CAG CGC GGG GCG GCC ACT TCT CCT GTG CCT CCG CCC GCT GCT-3' (SEQ ID NO: 2);  
 5'-GCC GCG CGG ATG CCA AGC ACC AGC TTT CCA GTC CCT TCC AAG-3' (SEQ ID NO: 3);  
 5'-CCA ACG TCA GCC CCG CCC TGC CGC TCC CCA CGG CGC ACT CCA-3' (SEQ ID NO: 4);  
 5 5'-TTC AGA CCT CCA CAC CGG GCA TCA TCC CGC CGG CGG-3' (SEQ ID NO: 5);  
 5'-GCC ACA CCA GGC CTG ATG GGG CCC CTG CCC TGG AGA GTC CTC-3' (SEQ ID NO: 6);  
 5'-AGT CTG CCC AGC CTG GAG GCC TAC AGA GAC CCC TCG TGC CTG-3' (SEQ ID NO: 7);  
 5'-GTG TCT CCC AAG ACC ACG GAC CCC GAG GAG GGC TTT CCC-3' (SEQ ID NO: 8);  
 5'-AGC TGG CTG GGT GCC CGC TCC TCC AGA CCC GCG TCC CCT TGC-3' (SEQ ID NO: 9);  
 10 5'-TAC AGC CTC AAC GGC CGG CAG CCG CCC TAC TCA CCC CAC CAC-3' (SEQ ID NO: 10);  
 5'-GAC CAC CGA CAG CAG CCT GGA CCT GGG AGA TGG CGT CCC TGT-3' (SEQ ID NO: 11);  
 5'-CCT GGG CAG CCC CCC GCC CCC GGC CGA CTT CGC GCC CGA AGA-3' (SEQ ID NO: 12);  
 5'-GCT CCC CTA CCA GTG GCG AAG CCC AAG CCC CTG TCC CCT ACG-3' (SEQ ID NO: 13);  
 5'-CTT CGG ATT GAG GTG CAG CCC AAG TCC CAC CAC CGA GCC CAC-3' (SEQ ID NO: 14);  
 15 5'-CAT GGC TAC TTG GAG AAT GAG CCG CTG ATG CTG CAG CTT TTC-3' (SEQ ID NO: 15);  
 5'-AAG ACC GTG TCC ACC ACC AGC CAC GAG GCT ATC CTC TCC AAC-3' (SEQ ID NO: 16);  
 5'-TCA GCT CAG GAG CTG CCT CTG GTG GAG AAG CAG AGC ACG GAC-3' (SEQ ID NO: 17);  
 5'-AAC GCC ATC TTT CTA ACC GTA AGC CGT GAA CAT GAG CGC G-3' (SEQ ID NO: 18);  
 5'-AGA AAC GAC GTC GCC GTA AAG CAG CGT GGC GTG TGG CA-3' (SEQ ID NO: 19); and  
 20 5'-GCA TAC TCA GAT AGT CAC GGT TAT TTT GCT TCT TGC GAA TG-3' (SEQ ID NO: 20).

Also for example but not limitation, the following pair of PCR primers (amplimers) may be used to amplify murine or human NF-AT<sub>c</sub> sequences (e.g., by reverse transcriptase initiated PCR of RNA from NF-AT<sub>c</sub> expressing cells):

25 (forward) 5'-AGGGCGCGGGCACCGGGCGCGGCAGGGCTCGGAG-3' (SEQ ID NO: 21)  
 (reverse) 5'-GCAAGAAGCAAAATAACCGTGACTATCTGAGTATGC-3' (SEQ ID NO: 22)

If desired, PCR amplimers for amplifying substantially full-length cDNA copies may be 30 selected at the discretion of the practitioner. Similarly, amplimers to amplify single NF-AT<sub>c</sub> exons or portions of the NF-AT<sub>c</sub> gene (murine or human) may be selected.

Each of these sequences may be used as hybridization probes or PCR amplimers to detect the presence of NF-AT<sub>c</sub> mRNA, for example to diagnose a disease characterized by the presence of an elevated NF-AT<sub>c</sub> mRNA level in lymphocytes, or to perform tissue typing (i.e., identify 35 tissues characterized by the expression of NF-AT<sub>c</sub> mRNA), and the like. The sequences may also be used for detecting genomic NF-AT<sub>c</sub> gene sequences in a DNA sample, such as for forensic DNA analysis (e.g., by RFLP analysis, PCR product length(s) distribution, etc.) or for diagnosis of diseases characterized by amplification and/or rearrangements of the NF-AT<sub>c</sub> gene.

Disclosure of the full coding sequence for human NF-AT<sub>c</sub> shown in Fig. 1 makes possible 40 the construction of isolated polynucleotides that can direct the expression of NF-AT<sub>c</sub> fragments

thereof, or analogs thereof. Further, the sequences in Fig. 1 make possible the construction of nucleic acid hybridization probes and PCR primers that can be used to detect RNA and DNA sequences encoding NF-AT<sub>c</sub>.

NF-AT polynucleotides of the invention include full-length NF-AT polynucleotides or portions thereof. In one embodiment, the NF-AT polynucleotide is at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, more preferably at least about 98% and most preferably at least about 99% identical to a nucleotide sequence shown in Figure 1, or set forth in SEQ ID NO: 45, or other exemplary NF-AT sequences, or of a portion thereof. Accordingly, the invention comprises polynucleotides encoding NF-ATc family members other than NF-ATc having SEQ ID NO: 38, which is referred to herein as NF-ATc1. Such family members include NF-ATc2 (GenBank I38152, U43341, U43342; also referred to as NF-ATp), NF-ATc3 (GenBank L41067, I38155, I38156; also referred to as NF-AT4 or NF-ATx), and NF-ATc4 (GenBank L41066, I38154; also referred to as NF-AT3) as well as differentially spliced forms thereof, as described, e.g., in U.S. Patent No. 5,612,455 issued to Hoey on March 18, 1997 and 5,656,452, issued to Rao et al., on August 12, published PCT WO 95/02035 by Arai et al. and WO 94/15964 by Rao et al.

Preferred portions or fragments of NF-ATc proteins include those comprising one or more specific domains. At least the following NF-ATc domains have been identified: a DNA-binding domain, corresponding essentially to the Rel Homology Domain (RHD) or Rel Similary Domain (RSD); a domain interacting with another protein, e.g., AP-1 or a target site of PKA or GSK-3; a nuclear localization sequence (NLS), e.g., comprising amino acids 265-267 of SEQ ID NO: 38 (N-terminal NLS) or amino acids 681-685 of SEQ ID NO: 38 (C-terminal NLS), or a domain interacting with an NLS, e.g., SRR (amino acids 172-194 of SEQ ID NO: 38), SP1 (amino acids 199-219 of SEQ ID NO: 38), SP2 (amino acids 233-252 of SEQ ID NO: 38), and SP3 (amino acids 278-301 of SEQ ID NO: 38). Other potential domains can be identified as further described herein. Accordingly, the invention provides NF-ATc polynucleotides encoding portions of NF-ATc polypeptides capable of exercising (agonists) or inhibiting (antagonists) at least one biological activity of NF-ATc, e.g., binding to another molecule, such as DNA or another protein, translocating across the nuclear membrane of a cell, or inhibiting translocation across a nuclear membrane of a cell. Assays for confirming that an NF-AT polypeptide is an agonist or an antagonist of a specific biological activity of NF-AT are further described herein.

Other preferred nucleic acids of the invention encode an NF-ATc polypeptide or portion thereof, e.g., a portion corresponding to a certain domain or having a specific biological activity and polypeptides which are at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, more preferably at least about 98% and most preferably at least about 99% identical or similar to a portion of human NF-ATc set forth in SEQ ID NO: 38, or other exemplary NF-AT sequences. For example, a preferred nucleic acid of the invention encodes an NF-AT polypeptide which is capable of

modulating translocation across the nuclear membrane. The NF-AT polypeptide can include an NLS or a region of NF-ATc interacting therewith, such as a domain selected from the group consisting of SRR, SP1, SP2, and SP3. Furthermore, the polynucleotides of the invention can encode wild-type or mutated forms of NF-ATc polypeptides, such as those described in the 5 Examples. For example, preferred polynucleotides encode an NF-ATc polypeptide having one or more serine that has been substituted with another amino acid, e.g., an alanine, to thereby prevent its phosphorylation. Preferred polypeptides encoded by the nucleic acids of the invention are further described herein, e.g., in the section pertaining to NF-AT polypeptides.

Polynucleotides encoding full-length NF-AT<sub>c</sub> or fragments or analogs thereof, may include 10 sequences that facilitate transcription (expression sequences) and translation of the coding sequences, such that the encoded polypeptide product is produced. Construction of such polynucleotides is well known in the art and is described further in Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd Ed. (1989), Cold Spring Harbor, N.Y. For example, but not for limitation, such polynucleotides can include a promoter, a transcription termination site 15 (polyadenylation site in eukaryotic expression hosts), a ribosome binding site, and, optionally, an enhancer for use in eukaryotic expression hosts, and, optionally, sequences necessary for replication of a vector. A typical eukaryotic expression cassette will include a polynucleotide sequence encoding a NF-AT<sub>c</sub> polypeptide linked downstream (i.e., in translational reading frame orientation; polynucleotide linkage) of a promoter such as the HSV *tk* promoter or the *pgk* 20 (phosphoglycerate kinase) promoter, optionally linked to an enhancer and a downstream polyadenylation site (e.g., an SV40 large T Ag poly A addition site).

A preferred NF-AT<sub>c</sub> polynucleotide encodes a NF-AT<sub>c</sub> polypeptide that comprises at least one of the following amino acids sequences:

- NAIFLTVSREHERVGC- (SEQ ID NO: 25);
- LHGYLENEPLMLQLFIGT- (SEQ ID NO: 26);
- PSTSPPRASVTEESWLG- (SEQ ID NO: 27);
- GPAPRAGGTMKSAEEEHYG- (SEQ ID NO: 28);
- ASAGGHPIVQ- (SEQ ID NO: 29);
- NTRVRLVFRV- (SEQ ID NO: 30);
- AKTDRDLCKPNSLVVEIPPFRN- (SEQ ID NO: 31);
- EVQPKSHHRAHYETEGSR- (SEQ ID NO: 32);
- SPRVSVTDDSWLGNT- (SEQ ID NO: 33);
- SHHRAHYETEGSRGAV- (SEQ ID NO: 34);
- LRNSDIELRKGETDGR- (SEQ ID NO: 35); and
- TLSLQVASNPIEC- (SEQ ID NO: 36).

The degeneracy of the genetic code gives a finite set of polynucleotide sequences encoding these amino acid sequences; this set of degenerate sequences may be readily generated by hand or by computer using commercially available software (Wisconsin Genetics Software Package Relaes 7.0). Thus, isolated polynucleotides typically less than approximately 10,000 nucleotides in length and comprising sequences encoding each of the following amino acid sequences:

- 5 -NAIFLTVSREHERVGC- (SEQ ID NO: 25);
- LHGYLENEPLMLQLFIGT- (SEQ ID NO: 26);
- PSTSPRASVTEESWLG- (SEQ ID NO: 27);
- GPAPRAGGTMKSAEEEHYG- (SEQ ID NO: 28);
- 10 -ASAGGHPIVQ- (SEQ ID NO: 29);
- NTRVRLVFRV- (SEQ ID NO: 30);
- AKTDRDLCKPNSLVVEIPPFRN- (SEQ ID NO: 31);
- EVQPCKSHRAHYETEGSR- (SEQ ID NO: 32);
- SPRVSVTDDSWLGNT- (SEQ ID NO: 33);
- 15 -SHHRAHYETEGSRGAV- (SEQ ID NO: 34);
- LRNSDIELRKGETDGR- (SEQ ID NO: 35); and
- TLSIQLVASNPIEC- (SEQ ID NO: 36).

are provided and may be used for, among other uses, the expression of a NF-AT<sub>c</sub> polypeptide which can be used as an immunogen, immunological reagent, and the like. Such polynucleotides 20 typically comprise an operably linked promoter for driving expression in a suitable prokaryotic or eukaryotic host cell. One exemplification of such a polynucleotide is the human NF-AT<sub>c</sub> cDNA sequence of Fig. 1 cloned in operable linkage to the mammalian expression vector pSR $\alpha$ , many alternative embodiments will be apparent to those of skill in the art, including the use of alternative expression vectors (e.g., pBC12BI and p91023(B); Hanahan J (1983) *J. Mol. Biol.* **166**: 577; 25 Cullen et al. (1985) *J. Virol.* **53**: 515; Lomedico PT (1982) *Proc. Natl. Acad. Sci. (U.S.A.)* **79**: 5798; Morinaga et al. (1984) *Bio/Technology* **2**: 636).

30 Additionally, where expression of a polypeptide is not desired, polynucleotides of this invention need not encode a functional protein. Polynucleotides of this invention may serve as hybridization probes and/or PCR primers (amplimers) and/or LCR oligomers for detecting NF-AT<sub>c</sub> RNA or DNA sequences.

35 Alternatively, polynucleotides of this invention may serve as hybridization probes or primers for detecting RNA or DNA sequences of related genes, such genes may encode structurally or evolutionarily related proteins. For such hybridization and PCR applications, the polynucleotides of the invention need not encode a functional polypeptide. Thus, polynucleotides of the invention may contain substantial deletions, additions, nucleotide substitutions and/or

transpositions, so long as specific hybridization or specific amplification to the NF-AT<sub>c</sub> sequence is retained.

Specific hybridization is defined hereinbefore, and can be roughly summarized as the formation of hybrids between a polynucleotide of the invention (which may include substitutions, 5 deletions, and/or additions) and a specific target polynucleotide such as human NF-AT<sub>c</sub> mRNA so that a single band is identified corresponding to each NF-AT<sub>c</sub> isoform on a Northern blot of RNA prepared from T cells (i.e., hybridization and washing conditions can be established that permit detection of discrete NF-AT<sub>c</sub> mRNA band(s)). Thus, those of ordinary skill in the art can prepare polynucleotides of the invention, which may include substantial additions, deletions, 10 substitutions, or transpositions of nucleotide sequence as compared to sequences shown in Fig. 1, or other exemplary NF-AT sequences, and determine whether specific hybridization is a property of the polynucleotide by performing a Northern blot using RNA prepared from a T lymphocyte cell line which expresses NF-AT<sub>c</sub> mRNA and/or by hybridization to a NF-AT<sub>c</sub> DNA clone (cDNA or genomic clone).

15 Specific amplification is defined as the ability of a set of PCR amplimers, when used together in a PCR reaction with a NF-AT<sub>c</sub> polynucleotide, to produce substantially a single major amplification product which corresponds to a NF-AT<sub>c</sub> gene sequence or mRNA sequence. Generally, human genomic DNA or mRNA from NF-AT<sub>c</sub> expressing human cells (e.g., Jurkat cell 20 line) is used as the template DNA sample for the PCR reaction. PCR amplimers that exhibit specific amplification are suitable for quantitative determination of NF-AT<sub>c</sub> mRNA by quantitative PCR amplification. NF-AT<sub>c</sub> allele-specific amplification products, although having sequence and/or length polymorphisms, are considered to constitute a single amplification product for purposes of this definition.

Generally, hybridization probes comprise approximately at least 25 consecutive nucleotides 25 of a sequence shown in Fig. 1 (for human and murine NF-AT<sub>c</sub> detection, respectively), preferably the hybridization probes contain at least 50 consecutive nucleotides of a sequence shown in Fig. 1, and more preferably comprise at least 100 consecutive nucleotides of a sequence shown in Fig. 1. PCR amplimers typically comprise approximately 25 to 50 consecutive nucleotides of a sequence shown in Fig. 1, and usually consist essentially of approximately 25 to 50 consecutive 30 nucleotides of a sequence shown in Fig. 1 with additional nucleotides, if present, generally being at the 5' end so as not to interfere with polymerase-mediated chain extension. PCR amplimer design and hybridization probe selection are well within the scope of discretion of practitioners of ordinary skill in the art.

35 B. Antisense Polynucleotides

Additional embodiments directed to modulation of NF-AT activity include methods that employ specific antisense polynucleotides complementary to all or part of the sequences shown in Fig. 1, or other exemplary NF-AT sequences, as well as ribozymes and molecules forming triplex structures. Such complementary antisense polynucleotides may include nucleotide substitutions, additions, deletions, or transpositions, so long as specific hybridization to the relevant target sequence of the NF-AT gene is retained as a functional property of the polynucleotide. Complementary antisense polynucleotides include soluble antisense RNA or DNA oligonucleotides which can hybridize specifically to NF-AT<sub>c</sub> mRNA species and prevent transcription of the mRNA species and/or translation of the encoded polypeptide (Ching et al. 5 (1989) Proc. Natl. Acad. Sci. U.S.A. **86**: 10006; Broder et al. (1990) Ann. Int. Med. **113**: 604; Loreau et al. (1990) FEBS Letters **274**: 53; Holcnenberg et al., WO91/11535; U.S.S.N. 07/530,165; WO91/09865; WO91/04753; WO90/13641; and EP 386563, each of which is incorporated herein by reference). The antisense polynucleotides therefore inhibit production of NF-AT<sub>c</sub> polypeptides. Since NF-AT<sub>c</sub> protein expression is associated with T lymphocyte activation, antisense 10 polynucleotides that prevent transcription and/or translation of mRNA corresponding to NF-AT<sub>c</sub> polypeptides may inhibit T cell activation and/or reverse the activated phenotype of T cells. Compositions containing a therapeutically effective dosage of NF-AT<sub>c</sub> antisense polynucleotides may be administered for treatment of immune diseases, including lymphocytic leukemias, and for inhibition of transplant rejection reactions, if desired. Likewise, NF-AT Antisense can be used to 15 inhibit NF-AT-mediated growth of cardiac and/or vascular tissues, e.g., as part of a treatment for cardiac hypertrophy. Antisense polynucleotides of various lengths may be produced, although such antisense polynucleotides typically comprise a sequence of about at least 25 consecutive nucleotides which are substantially identical to a naturally-occurring NF-AT<sub>c</sub> polynucleotide sequence, and typically which are identical to a sequence shown in Fig. 1, or other exemplary NF- 20 AT sequences.

Antisense polynucleotides may be produced from a heterologous expression cassette in a transfectant cell or transgenic cell, such as a transgenic pluripotent hematopoietic stem cell used to reconstitute all or part of the hematopoietic stem cell population of an individual. Alternatively, the antisense polynucleotides may comprise soluble oligonucleotides that are administered to the 25 external milieu, either in the culture medium *in vitro* or in the circulatory system or interstitial fluid *in vivo*. Soluble antisense polynucleotides present in the external milieu have been shown to gain access to the cytoplasm and inhibit translation of specific mRNA species. In some embodiments the antisense polynucleotides comprise methylphosphonate moieties. For general methods relating to antisense polynucleotides, see Antisense RNA and DNA, (1988), D.A. Melton, 30 Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

C. Isolation of the Cognate Human NF-AT<sub>c</sub> Gene

The human homolog of the NF-AT<sub>c</sub> cDNA is identified and isolated by screening a human genomic clone library, such as a human genomic library in yeast artificial chromosomes, cosmids, or bacteriophage  $\lambda$  (e.g.,  $\lambda$  Charon 35), with a polynucleotide probe comprising a sequence of 5 about at least 24 contiguous nucleotides (or their complement) of the cDNA sequence shown in Fig. 1, or other exemplary NF-AT sequences. Typically, hybridization and washing conditions are performed at high stringency according to conventional hybridization procedures. Positive clones are isolated and sequenced. For illustration and not for limitation, a full-length polynucleotide corresponding to the sequence of Fig. 1 may be labeled and used as a hybridization 10 probe to isolate genomic clones from a human or murine genomic clone library in  $\lambda$ EMBL4 or  $\lambda$ GEM11 (Promega Corporation, Madison, Wisconsin); typical hybridization conditions for screening plaque lifts (Benton and Davis (1978) *Science* 196: 180) can be: 50% formamide, 5 x SSC or SSPE, 1-5 x Denhardt's solution, 0.1-1% SDS, 100-200  $\mu$ g sheared heterologous DNA or tRNA, 0-10% dextran sulfate, 1  $\times$  10<sup>5</sup> to 1  $\times$  10<sup>7</sup> cpm/ml of denatured probe with a specific activity 15 of about 1  $\times$  10<sup>8</sup> cpm/ $\mu$ g, and incubation at 42 °C for about 6-36 hours. Prehybridization conditions are essentially identical except that probe is not included and incubation time is typically reduced. Washing conditions are typically 1-3 x SSC, 0.1-1% SDS, 50-70 °C with change of wash solution at about 5-30 minutes.

Nonhuman NF-AT<sub>c</sub> cDNAs and genomic clones (i.e., cognate nonhuman NF-AT<sub>c</sub> genes) 20 can be analogously isolated from various nonhuman cDNA and genomic clone libraries available in the art (e.g., Clontech, Palo Alto, CA) by using probes based on the sequences shown in Fig. 1, with hybridization and washing conditions typically being less stringent than for isolation of human NF-AT<sub>c</sub> clones.

Polynucleotides comprising sequences of approximately at least 30-50 nucleotides, 25 preferably at least 100 nucleotides, corresponding to or complementary to an NF-AT mRNA can serve as PCR primers and/or hybridization probes for identifying and isolating germline NF-AT genes. These germline genes may be human or may be from a related mammalian species, preferably rodents or primates. Such germline genes may be isolated by various methods conventional in the art, including, but not limited to, by hybridization screening of genomic 30 libraries in bacteriophage  $\lambda$  or cosmid libraries, or by PCR amplification of genomic sequences using primers derived from the sequences shown in Fig. 1. Human genomic libraries are publicly available or may be constructed de novo from human DNA.

Genomic clones of NF-AT<sub>c</sub>, particularly of the murine cognate NF-AT<sub>c</sub> gene, may be used to construct homologous targeting constructs for generating cells and transgenic nonhuman 35 animals having at least one functionally disrupted NF-AT<sub>c</sub> allele, preferably homozygous for knocked out NF-AT<sub>c</sub> alleles. Guidance for construction of homologous targeting constructs may

be found in the art, including: Rahemtulla et al. (1991) *Nature* **353**: 180; Jasen et al. (1990) *Genes Devel.* **4**: 157; Koh et al. (1992) *Science* **256**: 1210; Molina et al. (1992) *Nature* **357**: 161; Grusby et al. (1991) *Science* **253**: 1417; Bradley et al. (1992) *Bio/Technology* **10**: 534, incorporated herein by reference). Homologous targeting can be used to generate so-called "knockout" mice, which 5 are heterozygous or homozygous for an inactivated NF-AT<sub>c</sub> allele. Such mice may be sold commercially as research animals for investigation of immune system development, neoplasia, T cell activation, signal transduction, drug screening, and other uses.

Chimeric targeted mice are derived according to Hogan, et al., *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory (1988) and *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed., IRL Press, Washington, D.C., 10 (1987) which are incorporated herein by reference. Embryonic stem cells are manipulated according to published procedures (*Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed., IRL Press, Washington, D.C. (1987); Zjilstra et al. (1989) *Nature* **342**:435; and Schwartzberg et al. (1989) *Science* **246**: 799, each of which is incorporated herein 15 by reference).

Additionally, a NF-AT<sub>c</sub> cDNA or genomic gene copy may be used to construct transgenes for expressing NF-AT<sub>c</sub> polypeptides at high levels and/or under the transcriptional control of transcription control sequences which do not naturally occur adjacent to the NF-AT<sub>c</sub> gene. For example but not limitation, a constitutive promoter (e.g., a HSV-*tk* or *pgk* promoter) or a cell-20 lineage specific transcriptional regulatory sequence (e.g., a CD4 or CD8 gene promoter/enhancer) may be operably linked to a NF-AT<sub>c</sub>-encoding polynucleotide sequence to form a transgene (typically in combination with a selectable marker such as a *neo* gene expression cassette). Such transgenes can be introduced into cells (e.g., ES cells, hematopoietic stem cells) and transgenic cells and transgenic nonhuman animals may be obtained according to conventional methods. 25 Transgenic cells and/or transgenic nonhuman animals may be used to screen for antineoplastic agents and/or to screen for potential immunomodulatory agents, as overexpression of NF-AT<sub>c</sub> or inappropriate expression of NF-AT<sub>c</sub> may result in a hyperimmune state or enhance graft rejection reactions.

30 D. Expression of NF-AT<sub>c</sub> Polynucleotides

The nucleic acid sequences of the present invention capable of ultimately expressing the desired NF-AT<sub>c</sub> polypeptides can be formed from a variety of different polynucleotides (genomic or cDNA, RNA, synthetic oligonucleotides, etc.) as well as by a variety of different techniques.

As stated previously, the DNA sequences will be expressed in hosts after the sequences 35 have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as

episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline resistance or hygromycin resistance, to permit detection and/or selection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362, which is incorporated herein by reference).

5        E. coli is one prokaryotic host useful particularly for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilis, and other Enterobacteriaceae, such as Salmonella, Serratia, and various Pseudomonas species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In 10 addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

15        Other microbes, such as yeast, may also be used for expression. Saccharomyces is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.

20        In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention (see, Winnacker, "From Genes to Clones," VCH Publishers, N.Y., N.Y. (1987), which is incorporated herein by reference). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact human proteins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, myeloma cell lines, Jurkat cells, etc. Expression vectors for these cells can 25 include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen et al. (1986) Immunol. Rev. 89: 49, which is incorporated herein by reference), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, adenovirus, bovine 30 papillomavirus, and the like. The vectors containing the DNA segments of interest (e.g., polypeptides encoding a NF-AT<sub>c</sub> polypeptide) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, CaCl transfection is commonly utilized for prokaryotic cells, whereas CaPO<sub>4</sub> treatment or electroporation may be used for other cellular hosts. (See, generally, Maniatis, et al., Molecular Cloning: A Laboratory 35 Manual, Cold Spring Harbor Press, (1982), which is incorporated herein by reference). Usually, vectors are episomes and are maintained extrachromosomally.

Expression of recombinant NF-AT<sub>c</sub> protein in cells, particularly cells of the lymphopoietic lineage, may be used to identify and isolate genes that are transcriptionally modulated, either positively or negatively, by the presence of NF-AT<sub>c</sub> protein. Such genes are typically initially identified as cDNA clones isolated from subtractive cDNA libraries, wherein RNA isolated from 5 cells expressing recombinant NF-AT<sub>c</sub> and RNA isolated from control cells (i.e., not expressing recombinant NF-AT<sub>c</sub>) are used to generate the subtractive libraries and screening probes. In such a manner, NF-AT<sub>c</sub>-dependent genes may be isolated. NF-AT-dependent genes (or their regulatory sequences operably linked to a reporter gene) may be used as a component of an *in vitro* transcription assay employing a NF-AT<sub>c</sub> polypeptide as a necessary component for efficient 10 transcription; such transcription assays may be used to screen for agents which inhibit NF-AT<sub>c</sub>-dependent gene transcription and are thereby identified as candidate immunomodulatory agents.

#### E. NF-AT<sub>c</sub> Polypeptides

The nucleotide and amino acid sequences shown in Fig. 1, and those of other exemplary 15 NF-AT genes, enable those of skill in the art to produce polypeptides corresponding to all or part of the full-length human NF-AT<sub>c</sub> polypeptide sequence. Such polypeptides may be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding NF-AT<sub>c</sub>, or fragments and analogs thereof. Alternatively, such polypeptides may be synthesized by chemical 20 methods or produced by *in vitro* translation systems using a polynucleotide template to direct translation. Methods for expression of heterologous proteins in recombinant hosts, chemical synthesis of polypeptides, and *in vitro* translation are well known in the art and are described further in Maniatis et al., Molecular Cloning: A Laboratory Manual (1989), 2nd Ed., Cold Spring Harbor, N.Y. and Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, CA.

25 Fragments or analogs of NF-AT<sub>c</sub> may be prepared by those of skill in the art. Preferred amino- and carboxy-termini of fragments or analogs of NF-AT<sub>c</sub> occur near boundaries of functional domains. For example, but not for limitation, such functional domains include: (1) domains conferring the property of binding to other NF-AT components (e.g., AP-1), (2) domains conferring the property of nuclear localization, and (3) domains conferring the property of 30 enhancing activation of T cells when expressed at sufficient levels in such cells. Additionally, such functional domains might include: (1) domains conferring the property of binding to RNA polymerase species, (2) domains having the capacity to directly alter local chromatin structure, which may comprise catalytic activities (e.g., topoisomerases, endonucleases) and/or which may comprise structural features (e.g., zinc fingers, histone-binding moieties), and (3) domains which 35 may interact with accessory proteins and/or transcription factors.

One method by which structural and functional domains may be identified is by comparison of the nucleotide and/or amino acid sequence data shown in Fig. 1, or other exemplary NF-AT genes, to public or proprietary sequence databases. Preferably, computerized comparison methods are used to identify sequence motifs or predicted protein conformation domains that occur in other proteins of known structure and/or function, such as the zinc fingers. For example, the NAD-binding domains of dehydrogenases, particularly lactate dehydrogenase and malate dehydrogenase, are similar in conformation and have amino acid sequences that are detectably homologous (*Proteins, Structures and Molecular Principles*, (1984) Creighton (ed.), W.H. Freeman and Company, New York, which is incorporated herein by reference). Further, a method to identify protein sequences that fold into a known three-dimensional structure are known (Bowie et al. (1991) *Science* 253: 164). Thus, the foregoing examples demonstrate that those of skill in the art can recognize sequence motifs and structural conformations that may be used to define structural and functional domains in the NF-AT<sub>c</sub> sequences of the invention. One example of a domain is the *rel* similarity region from amino acid 418 to amino acid 710 of the NF-AT<sub>c</sub> polypeptide sequence of Fig. 1.

Additionally, computerized comparison of sequences shown in Fig. 1 to existing sequence databases can identify sequence motifs and structural conformations found in other proteins or coding sequences that indicate similar domains of the NF-AT<sub>c</sub> protein. For example but not for limitation, the programs GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package (Genetics Computer Group, 575 Science Dr., Madison, WI) can be used to identify sequences in databases, such as GenBank/EMBL, that have regions of homology with a NF-AT<sub>c</sub> sequences. Such homologous regions are candidate structural or functional domains. Alternatively, other algorithms are provided for identifying such domains from sequence data. Further, neural network methods, whether implemented in hardware or software, may be used to: (1) identify related protein sequences and nucleotide sequences, and (2) define structural or functional domains in NF-AT<sub>c</sub> polypeptides (Brunak et al. (1991) *J. Mol. Biol.* 220: 49, which is incorporated herein by reference). For example, the 13-residue repeat motifs -SPRASVTEESWLG- (SEQ ID NO: 23) and -SPRVSVTDDSWLG- (SEQ ID NO: 24) are examples of structurally related domains.

Fragments or analogs comprising substantially one or more functional domain may be fused to heterologous polypeptide sequences, wherein the resultant fusion protein exhibits the functional property(ies) conferred by the NF-AT<sub>c</sub> fragment. Alternatively, NF-AT<sub>c</sub> polypeptides wherein one or more functional domain have been deleted will exhibit a loss of the property normally conferred by the missing fragment.

By way of example and not limitation, the domain conferring the property of nuclear localization and/or interaction with AP-1 may be fused to  $\beta$ -galactosidase to produce a fusion

protein that is localized to the nucleus and which can enzymatically convert a chromogenic substrate to a chromophore.

5 Although one class of preferred embodiments are fragments having amino- and/or carboxy-termini corresponding to amino acid positions near functional domains borders, alternative NF-AT<sub>c</sub> fragments may be prepared. The choice of the amino- and carboxy-termini of such fragments rests with the discretion of the practitioner and will be made based on experimental considerations such as ease of construction, stability to proteolysis, thermal stability, immunological reactivity, amino- or carboxyl-terminal residue modification, or other considerations.

10 In addition to fragments, analogs of NF-AT<sub>c</sub> can be made. Such analogs may include one or more deletions or additions of amino acid sequence, either at the amino- or carboxy-termini, or internally, or both; analogs may further include sequence transpositions. Analogs may also comprise amino acid substitutions, preferably conservative substitutions. Additionally, analogs may include heterologous sequences generally linked at the amino- or carboxy-terminus, wherein the heterologous sequence(s) confer a functional property to the resultant analog which is not 15 indigenous to the native NF-AT<sub>c</sub> protein. However, NF-AT<sub>c</sub> analogs must comprise a segment of 25 amino acids that has substantial similarity to a portion of the amino acid sequence shown in Fig. 1, respectively, and which has at least one of the requisite functional properties enumerated in the Definitions (*supra*). Preferred amino acid substitutions are those which: (1) reduce 20 susceptibility to proteolysis, (2) reduce susceptibility to oxidation, (3) alter post-translational modification of the analog, possibly including phosphorylation, and (4) confer or modify other physicochemical or functional properties of such analogs, possibly including interaction with calcineurin or phosphorylation or dephosphorylation thereby. NF-AT<sub>c</sub> analogs include various 25 muteins of a NF-AT<sub>c</sub> sequence other than the naturally-occurring peptide sequence. For example, single or multiple amino acid substitutions (preferably conservative amino acid substitutions) may be made in the naturally-occurring NF-AT<sub>c</sub> sequence (preferably in the portion of the polypeptide outside the functional domains).

30 Conservative amino acid substitution is a substitution of an amino acid by a replacement amino acid which has similar characteristics (e.g., those with acidic properties: Asp and Glu). A conservative (or synonymous) amino acid substitution should not substantially change the structural characteristics of the parent sequence (e.g., a replacement amino acid should not tend to break a helix that occurs in the parent sequence, or disrupt other types of secondary structure that characterizes the parent sequence). Examples of art-recognized polypeptide secondary and 35 tertiary structures are described in Proteins, Structures and Molecular Principles, (1984) Creighton (ed.), W.H. Freeman and Company, New York; Introduction to Protein Structure, (1991), C. Branden and J. Tooze, Garland Publishing, New York, NY; and Thornton et al. (1991) Nature 354: 105; which are incorporated herein by reference).

The invention further provides phosphorylated NF-AT polypeptides. Preferred phosphorylated polypeptides comprise at least one phosphoserine, which can be selected from the group consisting of serines located in the region from about amino acid 172 to about amino acid 301. Even more preferred NF-AT polypeptides comprise a phosphorylated serine in SRR, SP1, 5 SP2, and/or SP3. Preferred serines in SRR include those at residues 172, 175, 176, 178, 179, 181, 184, 187, 188, 192, and 194 of SEQ ID NO: 38. Preferred serines in SP1 include those at residues 199, 203, 207, and 211 of SEQ ID NO: 38. Preferred serines in SP2 include those at residues 233, 327, and 245 of SEQ ID NO: 38. Preferred serines in SP3 include those at residues 278, 282, 286, 290, and 299 of SEQ ID NO: 38. Another preferred NF-AT polypeptide has a phosphoserine at 10 position 269.

The invention also provides peptides and peptidomimetics, e.g., for use in modulating nuclear translocation of an NF-AT protein. In a preferred embodiment, the agent comprises a portion of an NF-AT protein that is involved in translocation of an NF-AT polypeptide across the nuclear membrane of a cell, e.g., a portion of an NF-AT polypeptide that forms an intramolecular 15 association. In an even more preferred embodiment, the portion of NF-AT comprises a nuclear localization signal or sequence (NLS), such as the amino acid sequence KRKK (SEQ ID NO: 56) or KRKR (SEQ ID NO: 65) (referred to herein as KRKK/R (SEQ ID NO: 66)), corresponding to amino acids 682 to 685 of NF-ATc1 (SEQ ID NO: 38), or the amino acid sequence GKRKK/R (SEQ ID NO: 67), corresponding to amino acids 681-685 of SEQ ID NO: 38 or homologous 20 sequences in other NF-ATc family members. In fact, this C-terminal NLS, is also found in the other NF-ATc family members (see, e.g., Hoey et al. (1995) *Immunity* 2:461): NF-ATp (NF-ATc2) C-terminal NLS has the sequence NGKRKRS (SEQ ID NO: 68) (see figure 4); NF-ATc3 (NF-AT4) C-terminal NLS has the sequence NGKRKKS (SEQ ID NO: 69); and NF-ATc4 (NF-AT3) C-terminal NLS has the sequence NGRRKRS (SEQ ID NO: 70) (see Hoey et al., *supra*). 25 Accordingly, the invention provides peptides or peptidomimetics comprising these NLS. In another embodiment, the invention provides peptides and peptidomimetics comprising a nuclear localization signal including the amino acid sequence KRK, corresponding to amino acids 265-267 of NF-ATc1 (SEQ ID NO: 38). Alternatively, the peptide comprises the amino acid sequence CNKRKYSLN (SEQ ID NO: 53), corresponding to amino acids 263-271 of SEQ ID NO: 38.

30 The presence in a cell expressing NF-AT of a peptide comprising an NF-AT NLS will competitively inhibit the interaction of the endogenous NF-AT NLS with the SRR, SP1, SP2 and/or SP3 regions of NF-AT, thereby uncovering the NLS and allowing NF-AT to be translocated to the nucleus. Thus, the NF-AT NLS peptides of the invention constitute specific activators of NF-AT. In view of the sequence similarities between the NF-ATc family members, the 35 intramolecular interaction between NLSs and other domains of the NF-ATc1 molecule set forth herein, are believed to occur in the other NF-ATc family members and to regulate their

translocation across a nuclear membrane. The effect of these activators in a cell can be reversed by, e.g., introducing into the cell a peptide that is capable of interacting with an NF-AT NLS. For example, if a peptide comprising the NLS KRKK/R (SEQ ID NO: 66) is administered to a subject, the administration of a peptide capable of binding to this NLS will inhibit the activator.

5 Accordingly, the invention also provides peptides and peptidomimetics that are capable of interacting with an NLS in an NF-ATc molecule. Such peptides can be used, e.g., to stimulate translocation of NF-AT from the nucleus to the cytoplasm, by interacting with, and thereby shielding, the NLS of NF-AT molecules. Preferred peptides include N-terminal peptides, e.g., peptides located in the region from about amino acid 172-301 of SEQ ID NO: 38. Even more 10 preferred peptides comprise one or more sequence from the group consisting of SRR, SP1, SP2, and SP3. In one embodiment, such a peptide comprises an amino acid sequence of an SRR sequence, e.g., corresponding to about amino acids 172-194 of SEQ ID NO: 38. Shorter peptides can also be used so long as they are capable of interacting with the NLS. Other peptides that can be used for this purpose include peptides comprising one or more sequences selected from the 15 group consisting of SP1, SP2 and SP3, e.g., peptides comprising about amino acids 199-219 of SEQ ID NO: 38 (corresponding to SP1), about amino acids 233-252 of SEQ ID NO: 38 (SP2), and/or amino acids 278-301 of SEQ ID NO: 38 (SP3). Other peptides comprising amino acid sequence that are homologous (at least about 80%, 85%, 90%, 95%, or preferably at least about 98% or 99% identical or similar) to the SRR, SP1, SP2, and SP3 sequences set forth above are also 20 within the scope of the invention. In particular, peptides from NF-ATc2, NF-ATc3 and NF-ATc5 that are capable of forming intramolecular interactions with an NLS are part of the invention. SP1, SP2 and SP3 sequences are homologous in all NF-ATc family members (see, e.g., Hoey et al., *supra*).

25 The NF-AT peptides comprising one or more sequences selected from the group of SRR, SP1, SP2, and SP3 preferably comprise phosphoserines. Each serine of the peptide can be phosphorylated. However, as indicated in the Examples, it is only necessary to have some serines phosphorylated (see Figure 12) so that the peptide will interact with an NLS. Thus, preferred peptides of the invention are NF-AT peptides having a number of phosphoserines sufficient to allow the peptide to interact with an NLS of NF-AT. A peptide of interest can be phosphorylated 30 *in vitro* according to the method described in the Examples or according to *in vitro* phosphorylation assays known in the art.

35 The invention also provides NF-AT peptides comprising an NLS or one or more of the repeats SRR, SP1, SP2 and SP3 which are homologs, variants, derivatives or peptidomimetics of sequences set forth in SEQ ID NO: 38. Preferred homologs, variants, derivatives, or peptidomimetics are capable of interacting with a portion of an NF-AT polypeptide. Peptides or

peptidomimetics can be screened for those that interact with a portion of NF-AT using a binding assay, e.g., the binding assay described in the Examples.

Also within the scope of the invention are NF-AT polypeptides which are constitutively active. Such NF-T polypeptides can be useful since they are not dependent on the presence of calcium to be activated, but rather they can be activated in a regulated manner of choice, as further described below. As shown in the examples, the mutation of certain amino acids in NF-AT result in constitutive nuclear localization and thus constitutive activity. Preferred constitutively active NF-AT polypeptides have at least one amino acid deletion, addition or substitution (generally referred to as "peptide modification") that interferes in the intramolecular interactions in NF-AT.

Even more preferred NF-AT polypeptides have a peptide modification located in one or more of the SRR, SP1, SP2 or SP3 sequences of an NF-AT molecule, such that the ability of an NF-AT polypeptide to form an intramolecular association is decreased or inhibited. The peptide modification can be a substitution of one or more of the serines in one or more of the repeats. For example, a constitutively active NF-AT peptide can have a substitution of all the serines located in the SRR region (amino acid 172 to amino acid 194 of SEQ ID NO: 38). Alternatively, a constitutively active peptide can have a substitution of the serines at positions 184, 187 and 188 of SEQ ID NO: 38; a substitution of the serines at positions 172, 175, and 176 of SEQ ID NO: 38; a substitution of the serines at position 178, 179, and 181 of SEQ ID NO: 38; or a substitution of the serines at position 184, 187, and 188 of SEQ ID NO: 38. Constitutively active NF-AT polypeptides can also be obtained by the substitution of one or more serine in the SP1, SP2, and/or SP3 domains. In particular, constitutive NF-AT peptides can comprise a substitution of the four serines in SP1 (corresponding to amino acids 199-219 of SEQ ID NO: 38); substitution of the serines at position 233 and 237 of SP2 (corresponding to amino acids 233-252 of SEQ ID NO: 38); or substitution of serines at 278, 282, 286, and 299 in SP3 (corresponding to amino acids 278-301 of SEQ ID NO: 38). The one or more serines can be substituted with any amino acid so long as the substitution reduces intramolecular interactions, and is preferably an amino acid which cannot be phosphorylated, e.g., an alanine. Mutations that must be made in an NF-AT polypeptide to render it constitutively active can also be identified by screening a library of peptides. For example, one can produce a library of degenerate peptides, e.g., peptides comprising amino acids 172-188 of SEQ ID NO: 38, in which one or more serines or other amino acid is randomly mutated. This library can then be screened for those peptides which fail to interact with an NF-AT NLS, such as by passing the library of degenerate peptides over a column containing an excess of NF-AT NLS peptides. The amino acid sequence of the peptides in the flow-through of the column can then be determined. Alternatively, individual peptides in a library can be tagged, and their identity determined by detecting and identifying the tag.

Constitutively active NF-AT polypeptides can nevertheless be regulated, e.g., by having the gene encoding the constitutively active NF-AT polypeptide under the control of an inducible promoter. Alternatively, the NF-AT polypeptide can be fused to another peptide which can be regulated. For example, a constitutively active NF-AT polypeptide can be fused to a ligand binding domain. Control of the activity of this NF-AT protein can be obtained by further expressing in the cell a fusion protein comprising a ligand binding domain and a cytoplasmic retention domain, such that in the presence of a dimerizer molecule permitting cross-hybridization between the two fusion proteins, the NF-AT fusion protein is retained in the cytoplasm. Translocation to the nucleus is then induced by eliminating the dimerizer.

Alternatively, a constitutively active NF-AT polypeptide can be obtained by fusing the NF-AT polypeptide to an additional NLS, in particular a heterologous NLS, e.g., a viral NLS, such as the SV40 large T antigen NLS. An NF-AT polypeptide can also be fused with two or more NLS. The one or more NLS can be fused to the N-terminus of the NF-AT polypeptides. As shown in Example 10, an NF-AT polypeptide that is covalently linked to one or two copies of a heterologous NLS results in constitutive nuclear localization.

An NLS of NF-AT can also be used to direct a protein, in particular a heterologous protein to the nucleus. As shown in Example 11, the addition of a peptide having the amino acid sequence from amino acid 263-271 of SEQ ID NO: 38 (N-terminal NLS) or a peptide having the amino acid sequence from amino acid 681 to 685 of SEQ ID NO: 38 (C-terminal NLS) to a heterologous polypeptide resulted in constitutive nuclear localization of the polypeptide. Thus, the NLSs from NF-AT are sufficient to direct a polypeptide to the nucleus. Accordingly, also within the scope of the invention are peptides comprising an NLS from NF-AT, in particular, a peptide comprising the amino acid sequence KRK, or preferably CNKRKYSLN (SEQ ID NO: 53) (amino acids 263-271 of SEQ ID NO: 38), or even more preferably SPCNKRKYSLNGR (SEQ ID NO: 71) (amino acids 261-273 of SEQ ID NO: 38) and/or the amino acid sequence KRKK/R (SEQ ID NO: 66), or preferably GKRKK/R (SEQ ID NO: 67) (amino acids 681-685 of SEQ ID NO: 38), or even more preferably CNGKRKK/RSQ (SEQ ID NO: 72) (amino acids 679-687 of SEQ ID NO: 38).

A dominant negative NF-AT, or constitutively inactive NF-AT, can be produced by, e.g., mutating one, or preferably both NLS, such that the NF-AT polypeptide is incapable of translocating from the cytoplasm to the nucleus. An NF-AT polypeptide having one or more mutated NLS can act as a dominant negative mutant since, the polypeptide is still capable of interacting with calcineurin and thus, will compete for calcineurin, thereby prohibiting calcineurin to interact with the endogenous NF-AT molecules and activating them. The N-terminal NLS can be mutated, e.g., by substituting residues 265 to 268 (KRK) of SEQ ID NO: 38. For example, these residues can be changed to QIL. The C-terminal NLS can be mutated, e.g., by substituting residues

682-685 (KRKK/R (SEQ ID NO: 66)) of SEQ ID NO: 38. For example, these residues can be changed to TRTG (SEQ ID NO: 55). Other mutations are also within the scope of the invention and can be identified, e.g., by performing assays, as described in the examples. Such assays can also be used to screen libraries of mutated NLS sequences.

5       Particularly preferred variants are structural mimetics of a dominant negative NF-AT<sub>c</sub> mutants, such as a polypeptide consisting essentially of amino acids 1-418 of Fig. 1 and substantially lacking amino acids carboxy-terminal to residue 418. Such mimetics of dominant-negative mutant polypeptides can have substantial activity as antagonists or partial agonists of NF-AT activation (and hence T cell activation).

10      Still another aspect of the present invention relates to peptide and peptidomimetic inhibitors, derived from the NLS sequence, which inhibit glucan synthase kinases, e.g., GSK-3.

Native NF-AT<sub>c</sub> proteins, fragments thereof, or analogs thereof can be used as reagents in DNA binding assays and/or *in vitro* transcription assays for identifying agents that interfere with 15 NF-AT function, said agents are thereby identified as candidate drugs which may be used, for example, to block T cell activation or treat T cell lymphocytic leukemias. Typically, *in vitro* DNA binding assays that measure binding of NF-AT to DNA employ double-stranded DNA that contains an array of one or more NF-AT recognition sites (as defined by specific footprinting of native NF-AT protein). The DNA is typically linked to a solid substrate by any of various means 20 known to those of skill in the art; such linkage may be noncovalent (e.g., binding to a highly charged surface such as Nylon 66) or may be by covalent bonding (e.g., typically by chemical linkage involving a nitrogen position in a nucleotide base, such as diazotization). NF-AT<sub>c</sub> polypeptides are typically labeled by incorporation of a radiolabeled amino acid. The labeled NF-AT<sub>c</sub> polypeptide, usually reconstituted with an NF-AT nuclear component (e.g., AP-1 activity) to 25 form an NF-AT complex, is contacted with the immobilized DNA under aqueous conditions that permit specific binding in control binding reactions with a binding affinity of about  $1 \times 10^6 \text{ M}^{-1}$  or greater (e.g., 10-250 mM NaCl or KCl and 5-100 mM Tris HCl pH 5-9, usually pH 6-8), generally including Zn<sup>-2</sup> and/or Mn<sup>+2</sup> and/or Mg<sup>-2</sup> in the nanomolar to micromolar range (1 nM to 999  $\mu\text{M}$ ). Specificity of binding is typically established by adding unlabeled competitor at 30 various concentrations selected at the discretion of the practitioner. Examples of unlabeled protein competitors include, but are not limited to, the following: unlabeled NF-AT<sub>c</sub> polypeptide, bovine serum albumin, and nuclear protein extracts. Binding reactions wherein one or more agents are added are performed in parallel with a control binding reaction that does not include an agent. Agents which inhibit the specific binding of NF-AT<sub>c</sub> polypeptides to DNA, as compared to a 35 control reaction, are identified as candidate immunomodulatory drugs. Also, agents which prevent

transcriptional modulation by NF-AT *in vitro* are thereby identified as candidate immunomodulatory drugs.

As set forth above, in addition to NF-AT<sub>c</sub> polypeptides consisting only of naturally-occurring amino acids, NF-AT<sub>c</sub> peptidomimetics are also provided. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics" (Fauchere, J. (1986) Adv. Drug Res. **15**: 29; Veber and Freidinger (1985) TINS p.392; and Evans et al. (1987) J. Med. Chem. **30**: 1229, which are incorporated herein by reference) and are usually developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biological or pharmacological activity), such as human NF-AT<sub>c</sub>, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: -CH<sub>2</sub>NH-, -CH<sub>2</sub>S-, -CH<sub>2</sub>-CH<sub>2</sub>-, -CH=CH- (cis and trans), -COCH<sub>2</sub>-, -CH(OH)CH<sub>2</sub>-, and -CH<sub>2</sub>SO-, by methods known in the art and further described in the following references: Spatola, A.F. in "Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins," B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A.F., Vega Data (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review); Morley, J.S., Trends Pharm Sci (1980) pp. 463-468 (general review); Hudson, D. et al., Int J Pept Prot Res (1979) **14**:177-185 (-CH<sub>2</sub>NH-, CH<sub>2</sub>CH<sub>2</sub>-); Spatola, A.F. et al., Life Sci (1986) **38**:1243-1249 (-CH<sub>2</sub>S); Hann, M.M., J Chem Soc Perkin Trans I (1982) 307-314 (-CH-CH-, cis and trans); Almquist, R.G. et al., J Med Chem (1980) **23**:1392-1398 (-COCH<sub>2</sub>-); Jennings-White, C. et al., Tetrahedron Lett (1982) **23**:2533 (-COCH<sub>2</sub>-); Szelke, M. et al., European Appln. EP 45665 (1982) CA: **97**:39405 (1982) (-CH(OH)CH<sub>2</sub>-); Holladay, M.W. et al., Tetrahedron Lett (1983) **24**:4401-4404 (-C(OH)CH<sub>2</sub>-); and Hruby, V.J., Life Sci (1982) **31**:189-199 (-CH<sub>2</sub>S-); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is -CH<sub>2</sub>NH-. Such peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others. Labeling of peptidomimetics usually involves covalent attachment of one or more labels, directly or through a spacer (e.g., an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecules(s) (e.g., immunoglobulin superfamily molecules) to which the peptidomimetic binds to produce the therapeutic effect. Derivitization (e.g., labelling) of peptidomimetics should not substantially interfere with the

desired biological or pharmacological activity of the peptidomimetic. Peptidomimetics of NF-AT<sub>c</sub> may be used as competitive or noncompetitive agonists or antagonists of NF-AT<sub>c</sub> function. For example, a NF-AT<sub>c</sub> peptidomimetic administered to a stimulated T cell containing NF-AT<sub>c</sub> and may compete with the naturally-occurring NF-AT<sub>c</sub> and reduce NF-AT activity. Alternatively, an 5 NF-AT<sub>c</sub> peptidomimetic administered to a T cell lacking NF-AT<sub>c</sub> may induce T cell activation or the like.

Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) may be used to generate more 10 stable peptides. In addition, constrained peptides (including cyclized peptides) comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods known in the art (Rizo and Giersch (1992) Ann. Rev. Biochem. **61**: 387, incorporated herein by reference); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

The amino acid sequences of NF-AT<sub>c</sub> polypeptides identified herein will enable those of 15 skill in the art to produce polypeptides corresponding to NF-AT<sub>c</sub> peptide sequences and sequence variants thereof. Such polypeptides may be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding a NF-AT<sub>c</sub> peptide sequence, frequently as part of a larger polypeptide. Alternatively, such peptides may be synthesized by chemical methods. Methods for expression of heterologous proteins in recombinant hosts, chemical synthesis of polypeptides, and 20 *in vitro* translation are well known in the art and are described further in Maniatis et al., Molecular Cloning: A Laboratory Manual (1989), 2nd Ed., Cold Spring Harbor, N.Y.; Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, CA; Merrifield, J. (1969) J. Am. Chem. Soc. **91**: 501; Chaiken I.M. (1981) CRC Crit. Rev. Biochem. **11**: 255; Kaiser et al. (1989) Science **243**: 187; Merrifield, B. (1986) Science **232**: 342; Kent, S.B.H. (1988) Ann. Rev. Biochem. **57**: 957; and Offord, R.E. (1980) Semisynthetic Proteins, Wiley Publishing, which are incorporated herein by reference).

#### F. Production and Applications of $\alpha$ -NF-AT<sub>c</sub> Antibodies

Native NF-AT<sub>c</sub> proteins, fragments thereof, or analogs thereof, may be used to immunize 30 an animal for the production of specific antibodies. These antibodies may comprise a polyclonal antiserum or may comprise a monoclonal antibody produced by hybridoma cells. For general methods to prepare antibodies, see Antibodies: A Laboratory Manual, (1988) E. Harlow and D. Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, which is incorporated herein by reference.

35 For example but not for limitation, a recombinantly produced fragment of human NF-AT<sub>c</sub> can be injected into a rat along with an adjuvant following immunization protocols known to those

of skill in the art so as to generate an immune response. Typically, approximately at least 1-50  $\mu$ g of a NF-AT<sub>c</sub> fragment or analog is used for the initial immunization, depending upon the length of the polypeptide. Alternatively or in combination with a recombinantly produced NF-AT<sub>c</sub> polypeptide, a chemically synthesized peptide having a NF-AT<sub>c</sub> sequence (e.g., peptides exemplified in Table II, *infra*) may be used as an immunogen to raise antibodies which bind a NF-AT<sub>c</sub> protein, such as the native human NF-AT<sub>c</sub> polypeptide having the sequence shown essentially in Fig. 1 or the native human NF-AT<sub>c</sub> polypeptide isoform. Immunoglobulins which bind the recombinant fragment with a binding affinity of at least  $1 \times 10^7 \text{ M}^{-1}$  can be harvested from the immunized animal as an antiserum, and may be further purified by immunoaffinity chromatography or other means. Additionally, spleen cells are harvested from the immunized animal (typically rat or mouse) and fused to myeloma cells to produce a bank of antibody-secreting hybridoma cells. The bank of hybridomas can be screened for clones that secrete immunoglobulins which bind the recombinantly produced NF-AT<sub>c</sub> polypeptide (or chemically synthesized NF-AT<sub>c</sub> polypeptide) with an affinity of at least  $1 \times 10^6 \text{ M}^{-1}$ . Animals other than mice and rats may be used to raise antibodies; for example, goats, rabbits, sheep, and chickens may also be employed to raise antibodies reactive with a NF-AT<sub>c</sub> protein. Transgenic mice having the capacity to produce substantially human antibodies also may be immunized and used for a source of  $\alpha$ -NF-AT<sub>c</sub> antiserum and/or for making monoclonal-secreting hybridomas.

Bacteriophage antibody display libraries may also be screened for binding to a NF-AT<sub>c</sub> polypeptide, such as a full-length human NF-AT<sub>c</sub> protein, a NF-AT<sub>c</sub> fragment (e.g., a peptide having a sequence shown in Table II, *infra*), or a fusion protein comprising a NF-AT<sub>c</sub> polypeptide sequence of at least 14 contiguous amino acids as shown in Fig. 1 or a polypeptide sequence of Table II (*infra*). Combinatorial libraries of antibodies have been generated in bacteriophage lambda expression systems which may be screened as bacteriophage plaques or as colonies of lysogens (Huse et al. (1989) *Science* **246**: 1275; Caton and Koprowski (1990) *Proc. Natl. Acad. Sci. (U.S.A.)* **87**: 6450; Mullinax et al (1990) *Proc. Natl. Acad. Sci. (U.S.A.)* **87**: 8095; Persson et al. (1991) *Proc. Natl. Acad. Sci. (U.S.A.)* **88**: 2432). Various embodiments of bacteriophage antibody display libraries and lambda phage expression libraries have been described (Kang et al. (1991) *Proc. Natl. Acad. Sci. (U.S.A.)* **88**: 4363; Clackson et al. (1991) *Nature* **352**: 624; McCafferty et al. (1990) *Nature* **348**: 552; Burton et al. (1991) *Proc. Natl. Acad. Sci. (U.S.A.)* **88**: 10134; Hoogenboom et al. (1991) *Nucleic Acids Res.* **19**: 4133; Chang et al. (1991) *J. Immunol.* **147**: 3610; Breitling et al. (1991) *Gene* **104**: 147; Marks et al. (1991) *J. Mol. Biol.* **222**: 581; Barbas et al. (1992) *Proc. Natl. Acad. Sci. (U.S.A.)* **89**: 4457; Hawkins and Winter (1992) *J. Immunol.* **22**: 867; Marks et al. (1992) *Biotechnology* **10**: 779; Marks et al. (1992) *J. Biol. Chem.* **267**: 16007; Lowman et al (1991) *Biochemistry* **30**: 10832; Lerner et al. (1992) *Science* **258**: 1313, incorporated herein by reference). Typically, a bacteriophage antibody display library is screened

with a NF-AT<sub>c</sub> polypeptide that is immobilized (e.g., by covalent linkage to a chromatography resin to enrich for reactive phage by affinity chromatography) and/or labeled (e.g., to screen plaque or colony lifts).

NF-AT<sub>c</sub> polypeptides which are useful as immunogens, for diagnostic detection of  $\alpha$ -NF-AT<sub>c</sub> antibodies in a sample, for diagnostic detection and quantitation of NF-AT<sub>c</sub> protein in a sample (e.g., by standardized competitive ELISA), or for screening a bacteriophage antibody display library, are suitably obtained in substantially pure form, that is, typically about 50 percent (w/w) or more purity, substantially free of interfering proteins and contaminants. Preferably, these polypeptides are isolated or synthesized in a purity of at least 80 percent (w/w) and, more preferably, in at least about 95 percent (w/w) purity, being substantially free of other proteins of humans, mice, or other contaminants. Preferred immunogens comprise at least one NF-AT<sub>c</sub> polypeptide sequence shown in Table II, either as a discrete peptide or as part of a fusion polypeptide (e.g., with a  $\beta$ -galactosidase or glutathione S-transferase sequence). NF-AT<sub>c</sub> immunogens comprise at least one, typically several of such immunogenic epitopes.

For some applications of these antibodies, such as identifying immunocrossreactive proteins, the desired antiserum or monoclonal antibody(ies) is/are not monospecific. In these instances, it may be preferable to use a synthetic or recombinant fragment of NF-AT<sub>c</sub> as an antigen rather than using the entire native protein. More specifically, where the object is to identify immunocrossreactive polypeptides that comprise a particular structural moiety, such as a DNA-binding domain, it is preferable to use as an antigen a fragment corresponding to part or all of a commensurate structural domain in the NF-AT<sub>c</sub> protein. Production of recombinant or synthetic fragments having such defined amino- and carboxy-termini is provided by the NF-AT<sub>c</sub> sequences shown in Fig. 1.

If an antiserum is raised to a NF-AT<sub>c</sub> fusion polypeptide, such as a fusion protein comprising a NF-AT<sub>c</sub> immunogenic epitope fused to  $\beta$ -galactosidase or glutathione S-transferase, the antiserum is preferably preadsorbed with the non-NF-AT<sub>c</sub> fusion partner (e.g,  $\beta$ -galactosidase or glutathione S-transferase) to deplete the antiserum of antibodies that react (i.e., specifically bind to) the non-NF-AT<sub>c</sub> portion of the fusion protein that serves as the immunogen. Monoclonal or polyclonal antibodies which bind to the human and/or murine NF-AT<sub>c</sub> protein can be used to detect the presence of human or murine NF-AT<sub>c</sub> polypeptides in a sample, such as a Western blot of denatured protein (e.g., a nitrocellulose blot of an SDS-PAGE) obtained from a lymphocyte sample of a patient. Preferably quantitative detection is performed, such as by densitometric scanning and signal integration of a Western blot. The monoclonal or polyclonal antibodies will bind to the denatured NF-AT<sub>c</sub> epitopes and may be identified visually or by other optical means with a labeled second antibody or labeled Staphylococcus aureus protein A by methods known in

the art. Frequently, denatured NF-AT<sub>c</sub> will be used as the target antigen so that more epitopes may be available for binding.

Table II

### Selected Human NF-AT Antigen Peptides

10 -NAIFLTVSREHERVGC- (SEQ ID NO: 25);  
-LHGYLENEPLMLQLFIGT- (SEQ ID NO: 26);  
-PSTSPRASVTEESWLG- (SEQ ID NO: 27);  
-GPAPRAGGTMKSAEEEHYG- (SEQ ID NO: 28);  
-ASAGGHPIVQ- (SEQ ID NO: 29);  
-NTRVRLVFRV- (SEQ ID NO: 30);  
-AKTDRDLCKPNSLVVEIPPFRN- (SEQ ID NO: 31);  
-EVQPKSHHRAHYETEGSR- (SEQ ID NO: 32);  
-SPRVSVTDDSWLGNT- (SEQ ID NO: 33);  
-SHHRAHYETEGSRGAV- (SEQ ID NO: 34);  
-LRNSDIELRKGETDAGR- (SEQ ID NO: 35); and  
-TLSLQVASNPIEC- (SEQ ID NO: 36).

Such NF-AT<sub>c</sub> sequences as shown in Tables II may be used as an immunogenic peptide directly (e.g., to screen bacteriophage antibody display libraries or to immunize a rabbit), or may be conjugated to a carrier macromolecule (e.g., BSA) or may compose part of a fusion protein to be used as an immunogen. A preferred NF-AT<sub>c</sub> polypeptide comprises the following amino acids sequences:

25 -NAIFLTVSREHERVGC- (SEQ ID NO: 25);  
-PSTSPRASVTEESWLG- (SEQ ID NO: 27);  
-SPRVSVTDDSWLGNT- (SEQ ID NO: 33); and  
-SHHRAHYETEGSRGAV- (SEQ ID NO: 34);

and may comprise other intervening and/or terminal sequences; generally such polypeptides are less than 1000 amino acids in length, more usually less than about 500 amino acids in length; often spacer peptide sequences or terminal peptide sequences, if present, correspond to naturally occurring polypeptide sequences, generally mammalian polypeptide sequences. One application of the preferred NF-AT<sub>c</sub> polypeptide just recited is as a commercial immunogen to raise  $\alpha$ -NF-AT<sub>c</sub> antibodies in a suitable animal and/or as a commercial immunodiagnostic reagent for quantitative ELISA (e.g., competitive ELISA) or competitive RIA in conjunction with the anti-NF-AT<sub>c</sub> antibodies provided by the invention, such as for calibration or standardization of such immunoassays for staging or diagnosis of NF-AT<sub>c</sub>-expressing lymphocytic leukemias in humans or cell typing or identification of T cells (such as activated T cells and/or activatable T cells). The

preferred NF-AT<sub>c</sub> polypeptide just recited will find many other uses in addition to serving as an immunogen or immunological reagent. One or more of the above-listed sequences may be incorporated into a fusion protein with a fusion partner such as human serum albumin, GST, etc. For such fusion proteins in excess of 1000 amino acids, deletions in the fusion partner (albumin) 5 moiety may be made to bring the size to about 1000 amino acids or less, if desired.

In some embodiments, it will be desirable to employ a polyvalent NF-AT<sub>c</sub> antigen, comprising at least two NF-AT<sub>c</sub> immunogenic epitopes in covalent linkage, usually in peptide linkage. Such polyvalent NF-AT<sub>c</sub> antigens typically comprise multiple NF-AT<sub>c</sub> antigenic peptides from the same species (e.g., human or mouse), but may comprise a mix of antigenic peptides from 10 NF-AT<sub>c</sub> proteins of different species (i.e., an interspecies NF-AT<sub>c</sub> polyvalent antigen). Frequently, the spatial order of the antigenic peptide sequences in the primary amino acid sequence of a polyvalent antigen occurs in the same orientation as in the naturally occurring NF-AT<sub>c</sub> protein (i.e., a first antigenic peptide sequence that is amino-terminal to a second antigenic peptide sequence in a naturally occurring NF-AT<sub>c</sub> protein will be amino-terminal to said second antigenic peptide 15 sequence in a polyvalent antigen). Frequently, spacer peptide sequences will be used to link antigenic peptide sequences in a polyvalent antigen, such spacer peptide sequences may be predetermined, random, or pseudorandom sequences. Spacer peptide sequences may correspond to sequences known to be non-immunogenic to the animal which is to be immunized with the polyvalent antigen, such as a sequence to which the animal has been tolerized. Although many 20 examples of such polyvalent antigens may be given, the following embodiment is provided for illustration and not limitation:

-NAIFLTVSREHERVGC-(aa1) (SEQ ID NO: 25) -AKTDRDLCKPNSLVVEIPPFRN-(aa2) (SEQ ID NO: 31)-GILKLRNSDIELRKGETD- (SEQ ID NO: 37)

where (aa1) and (aa2) are peptide spacers of at least one amino acid and less than 1000 25 amino acids; aa1 is a peptide sequence selected independently from the aa2 peptide sequence; the length of aa1 (which may be composed of multiple different amino acids) is independent of the length of aa2 (which may be composed of multiple different amino acids).

Immunogenic NF-AT<sub>c</sub> peptides may be used to immunize an animal to raise anti-NF-AT<sub>c</sub> 30 antibodies and/or as a source of spleen cells for making a hybridoma library from which to select hybridoma clones which secrete a monoclonal antibody which binds to a NF-AT<sub>c</sub> protein with an affinity of  $1 \times 10^7 \text{ M}^{-1}$  or greater, preferably at least  $1 \times 10^8 \text{ M}^{-1}$  to  $1 \times 10^9 \text{ M}^{-1}$ . Such immunogenic NF-AT<sub>c</sub> peptides can also be used to screen bacteriophage antibody display libraries directly.

One use of such antibodies is to screen cDNA expression libraries, preferably containing 35 cDNA derived from human or murine mRNA from various tissues, for identifying clones containing cDNA inserts which encode structurally-related, immunocrossreactive proteins, that are candidate novel transcription factors or chromatin proteins. Such screening of cDNA

expression libraries is well known in the art, and is further described in Young et al., Proc. Natl. Acad. Sci. U.S.A. 80:1194-1198 (1983), which is incorporated herein by reference] as well as other published sources. Another use of such antibodies is to identify and/or purify immunocrossreactive proteins that are structurally or evolutionarily related to the native NF-AT<sub>c</sub> 5 protein or to the corresponding NF-AT<sub>c</sub> fragment (e.g., functional domain; DNA-binding domain) used to generate the antibody. It is believed that such antibodies will find commercial use as such reagents for research applications, just as other antibodies (and biological reagents - such as restriction enzymes and polymerases) are sold commercially.

Various other uses of such antibodies are to diagnose and/or stage leukemias or other 10 immunological disease states, and for therapeutic application (e.g., as cationized antibodies or by targeted liposomal delivery) to treat neoplasia, hyperimmune function, graft rejection, and the like.

An example of an NF-AT<sub>c</sub> polypeptide is a polypeptide having the sequence:

MPSTSFPVPSKFPLGAAA VFGRGETLGPAPRAGGTMKSAEEHYGYASSNVSPALPLPTAHS  
TLPAPCHNLQTSTPGIIPPADHPSGYGAALDGCPAGYFLSSGHTRPDGAPALESPRIEITSCL  
15 GLYHNNNQFFHDVEVEDVLPSKRSRSPSTATLSLPSLEAYRDPSCLS PASSLSSRSCNSEASSY  
ESNYSY PYASPQTSPWQSPCVSPKTTDPEEGFPRGLGACTLLGSPQHSPSTSPRASVTEESWL  
GARSSRPASPCNKRKYSLN GRQPPYSPHHSPTSPHGS PRVS VTDDSWLGNTTQYTSSAIVAA  
INALTTDSSL DLGDGV PVKSRK TTLEQPPSVALKVEPVGEDLGSPPPP ADFA PEDYSSFQHIR  
KGGFCDQYLA VPQHPYQWAKPKPLSPTSYMSPTLPA LDWQLPSHSGPYELRIEVQPKSHHRAH  
20 YETEGSRGAVK ASAGGHPIVQLHGYL ENEPLMLQLFIGTADD RLLRPHAFYQVHRITGKT VST  
TSHEA ILSNTKV LIEPLL PENS MRAV IDCACIL KLRNS DIELRK GETD IGRKNTRV RL VFRV H  
VPQPSGRTLSLQV ASNP IECS QRS A QELPL VEKQSTD SYPV VGGKKM VLS GHNFLQDSKVIFV  
EKAPDGHV WEMEAK TDRDLCKPNSL VVEIPPFRN QRITSPV HVS F YVCNGKR KRSQY QRFTY  
LPANGNAI FLTV SREHER VGCFF (SEQ ID NO: 38).

25 Other preferred antigens for preparing antibodies include phosphorylated NF-AT polypeptides or domains thereof. For example, the invention provides antibodies binding specifically to phosphorylated NF-AT<sub>c</sub> polypeptides, and not to those that are not phosphorylated. The NF-AT<sub>c</sub> polypeptide can be phosphorylated on a serine, such as a serine located in SRR, SP1, SP2, SP3 or which is located between these repetitive sequences. Such antibodies can be used to 30 hide or shield these phosphorylated domains from NLSs thereby favoring NF-AT<sub>c</sub> localization in the nucleus of a cell. Alternatively, such antibodies can be used to specifically detect the phosphorylated form, e.g., in diagnostics.

#### G. Identification and Isolation of Proteins That Bind NF-AT

35 Proteins that bind to NF-AT<sub>c</sub> and/or a NF-AT-DNA complex are potentially important transcriptional regulatory proteins. Such proteins may be targets for novel immunomodulatory

agents. These proteins are referred to herein as accessory proteins. Accessory proteins may be isolated by various methods known in the art.

One preferred method of isolating accessory proteins is by contacting a NF-AT<sub>c</sub> polypeptide to an antibody that binds the NF-AT<sub>c</sub> polypeptide, and isolating resultant immune complexes. These immune complexes may contain accessory proteins bound to the NF-AT<sub>c</sub> polypeptide. The accessory proteins may be identified and isolated by denaturing the immune complexes with a denaturing agent and, preferably, a reducing agent. The denatured, and preferably reduced, proteins can be electrophoresed on a polyacrylamide gel. Putative accessory proteins can be identified on the polyacrylamide gel by one or more of various well known methods (e.g., Coomassie staining, Western blotting, silver staining, etc.), and isolated by resection of a portion of the polyacrylamide gel containing the relevant identified polypeptide and elution of the polypeptide from the gel portion.

A putative accessory protein may be identified as an accessory protein by demonstration that the protein binds to NF-AT<sub>c</sub> and/or a NF-AT-DNA complex. Such binding may be shown *in vitro* by various means, including, but not limited to, binding assays employing a putative accessory protein that has been renatured subsequent to isolation by a polyacrylamide gel electrophoresis method. Alternatively, binding assays employing recombinant or chemically synthesized putative accessory protein may be used. For example, a putative accessory protein may be isolated and all or part of its amino acid sequence determined by chemical sequencing, such as Edman degradation. The amino acid sequence information may be used to chemically synthesize the putative accessory protein. The amino acid sequence may also be used to produce a recombinant putative accessory protein by: (1) isolating a cDNA clone encoding the putative accessory protein by screening a cDNA library with degenerate oligonucleotide probes according to the amino acid sequence data, (2) expressing the cDNA in a host cell, and (3) isolating the putative accessory protein. Alternatively, a polynucleotide encoding a NF-AT<sub>c</sub> polypeptide may be constructed by oligonucleotide synthesis, placed in an expression vector, and expressed in a host cell.

Putative accessory proteins that bind NF-AT<sub>c</sub> and/or NF-AT-DNA complex *in vitro* are identified as accessory proteins. Accessory proteins may also be identified by crosslinking *in vivo* with bifunctional crosslinking reagents (e.g., dimethylsuberimidate, glutaraldehyde, etc.) and subsequent isolation of crosslinked products that include a NF-AT<sub>c</sub> polypeptide. For a general discussion of cross-linking, see Kunkel et al. (1981) *Mol. Cell. Biochem.* 34: 3, which is incorporated herein by reference. Preferably, the bifunctional crosslinking reagent will produce crosslinks which may be reversed under specific conditions after isolation of the crosslinked complex so as to facilitate isolation of the accessory protein from the NF-AT<sub>c</sub> polypeptide. Isolation of crosslinked complexes that include a NF-AT<sub>c</sub> polypeptide is preferably accomplished

by binding an antibody that binds a NF-AT<sub>c</sub> polypeptide with an affinity of at least  $1 \times 10^7 \text{ M}^{-1}$  to a population of crosslinked complexes and recovering only those complexes that bind to the antibody with an affinity of at least  $1 \times 10^7 \text{ M}^{-1}$ . Polypeptides that are crosslinked to a NF-AT<sub>c</sub> polypeptide are identified as accessory proteins.

5      Screening assays can be developed for identifying candidate immunomodulatory agents as being agents which inhibit binding of NF-AT<sub>c</sub> to an accessory protein (e.g. AP-1) under suitable binding conditions (see *infra*).

10     Yeast two-hybrid systems may be used to screen a mammalian (typically human) cDNA expression library, wherein cDNA is fused to a GAL4 DNA binding domain or activator domain, and a NF-AT<sub>c</sub> polypeptide sequence is fused to a GAL4 activator domain or DNA binding domain, respectively. Such a yeast two-hybrid system can screen for cDNAs encoding proteins which bind to NF-AT<sub>c</sub> sequences. For example, a cDNA library can be produced from mRNA from a human mature T cell line or other suitable cell type. Such a cDNA library cloned in a yeast two-hybrid expression system (Chien et al. (1991) *Proc. Natl. Acad. Sci. (U.S.A.)* 88: 9578 or *Cell* 72: 233) 15 can be used to identify cDNAs which encode proteins that interact with NF-AT<sub>c</sub> and thereby produce expression of the GAL4-dependent reporter gene. Polypeptides which interact with NF-AT<sub>c</sub> can also be identified by immunoprecipitation of NF-AT<sub>c</sub> with antibody and identification of co-precipitating species. Further, polypeptides that bind NF-AT<sub>c</sub> can be identified by screening 20 a peptide library (e.g., a bacteriophage peptide display library, a spatially defined VLSIPS peptide array, and the like) with a NF-AT<sub>c</sub> polypeptide.

#### H      Exemplary diagnostic and prognostic methods of the invention

25     The invention provides diagnostic and prognostic methods, including methods for determining the state of immunosuppression in a subject and for determining an appropriate dose of immunosuppressant.

30     In one preferred embodiment of the invention, hybridization probes that specifically identify the NF-AT<sub>c</sub> gene may be used in methods for diagnosing genetic disease. For example, but not for limitation, the genetic disease thus diagnosed may involve a lesion in the relevant NF-AT<sub>c</sub> structural or regulatory sequences, or may involve a lesion in a genetic locus closely linked 35 to the NF-AT<sub>c</sub> locus and which can be identified by restriction fragment length polymorphism or DNA sequence polymorphism at the linked NF-AT<sub>c</sub> locus. In a further preferred embodiment, NF-AT<sub>c</sub> gene probes are used to diagnose or identify genetic disease involving predisposition to immunological disease, wherein the amount or functionality of endogenous NF-AT<sub>c</sub> is sufficient for the individual to exhibit an increased probability of developing an immune disease, particularly an immune deficiency, arthritis, or autoimmune disease.

The invention also provides a method for determining the state of immunosuppression of a subject. In one embodiment, the method comprises determining the level of cytoplasmic and/or nuclear NF-AT polypeptide in a cell, preferably a lymphocyte, of a subject. The method can comprise obtaining a blood sample from a subject, and determining the amount of cytoplasmic and/or nuclear NF-AT, such as by immunohistochemistry using an antibody that binds specifically to NF-AT, as further described herein. In a preferred embodiment, the method comprises incubating the blood cells of the subject with a T cell activating compound and/or calcium ionophore prior to determining the cellular localization of NF-AT. In fact, if a patient is immunosuppressed, stimulation of lymphocytes of the patient will not result in significant translocation of NF-AT into the nucleus. A preferred T cell activator is a polyclonal activator, including lectins, concanavalin-A (Con-A) and phytohemagglutinin (PHA). Other activators include antibodies binding to invariable framework epitopes on the T cell receptor or CD3.

A preferred method for detecting NF-AT is by immunofluorescence, using an antibody that binds specifically to one or more NF-ATc proteins. A preferred monoclonal antibody is the 7A6 antibody, described in Northrop et al.(1994) *Nature* 369:497.

In a preferred embodiment, the test is carried out on an enriched population of cells from the subject, such as a blood sample enriched in mononuclear cells. Peripheral blood mononuclear cells can be obtained, e.g., by separating the cells from a blood sample on a buffy coat, according to methods known in the art.

The level of nuclear and/or cytoplasmic NF-AT in lymphocytes of a subject can then be compared to the level of nuclear and/or cytoplasmic NF-AT in a control individual. A "control" or "normal" subject refers to a subject which has no known disease or condition involving NF-AT activation, e.g., inflammation, or autoimmune disease and which are not subjected to any treatment at the time the cell sample was obtained. Normal standards can be established from analysis of several cell samples from normal subjects. Samples from patients can then be analyzed and compared to this set of standards.

In a preferred embodiment, this diagnostic method can be used to monitor the state of immunosuppression in a subject who is receiving an immunosuppressive treatment, e.g., cyclosporin A. In one embodiment, cells are obtained from a subject, and the cellular localization of NF-AT is determined prior to and/or after incubation of the cells with a T cell activator, e.g., PHA. If the analysis reveals that the patient contains a high number of lymphocytes having nuclear NF-AT, i.e., activated lymphocytes, the patient should be given additional immunosuppressive drugs. Thus, a patient can be followed and an adequate amount of immunosuppressive drug can be administered to the patient, so that the patient does not receive excessive amounts of immunosuppressants, but received enough immunosuppressant to maintain him in an immunosuppressed state.

In another embodiment, the invention provides a method for determining the sensitivity of a subject to a particular immunosuppressive agent, e.g., cyclosporin A. In one embodiment, lymphocytes are obtained from a subject, the lymphocytes are incubated *in vitro* in the presence of various amounts of the immunosuppressive drug and for various times, and the cellular location of NF-AT is determined. The comparison of the results of this analysis with those obtained from the same analysis of lymphocytes from one or more normal subjects will indicate whether the subject is more, or alternatively, less sensitive, to the immunosuppressive drug than an average person. This analysis can also be performed *in vivo*. For example, a certain dose of immunosuppressive agent is administered to a subject and the cellular localization of NF-AT in lymphocytes of the subject is then determined at various time points and compared to that of a normal subject. Standards of normal subjects can also be obtained before hand and the results of the analysis can then be compared to these standards. Thus, based on the results of such tests, a physician can more appropriately predict the effective dose of immunosuppressant to administer to a subject, thereby avoiding administering an excessive of amount of immunosuppressant that could have toxic effects in the subject.

In other embodiments, the invention provides a method for determining the risk of a patient developing a disorder involving unwanted cardiovascular growth, or, if so diagnosed, identifying the etiology of the disorder. In the instance of the latter, understanding the molecular basis of the disease, including the role of an NF-AT protein in the disorder, can be useful to determine the proper course of therapy (i.e., would a particular therapy be effective or not), as well as proscribing a course of treatment based on NF-AT being a prognostic marker for reoccurrence, etc.

Such embodiments of the subject method include detecting changes to an NF-AT gene, e.g., point mutations, deletions, additions, chromosomal rearrangements, changes in methylation patterns, etc., as well as changes in the level of expression of the protein, the rate of turnover of the protein (ubiquitin-dependent or independent), the rate of phosphorylation/dephosphorylation, and/or the cellular localization of the protein.

#### I. Methods for drug design and screening assays

The invention further provides screening assays for identifying compounds which modulate the activity of NF-AT proteins. The screening assays can be *in vivo* or *in vitro* and can be cell based or based on a cell free format. These agents include, but are not limited to, compounds that either potentiate or inhibit an intrinsic activity of an NF-AT protein or a complex including an NF-AT protein, compounds that interfere with the interaction of the NF-AT protein with other protein(s) or nucleic acid, compounds which modify the rate of a certain post-translational modification of NF-AT proteins (e.g., by enzymes such as phosphatases or kinases), antisense constructs for inhibiting expression of NF-AT proteins, nucleic acid decoys for competitively

inhibiting binding of an NF-AT protein to a response element in genomic DNA, and compounds comprising forms of the NF-AT proteins that are altered (mutated) to provide dominant loss-of-function or gain-of-function activity. In one embodiment, the screening methods of the present invention are directed to the identification of agents capable of modulating, and in particular of 5 inhibiting, the development of hypertrophy of a cell, such as a muscle cell or which are capable of reducing hypertrophy of a cell.

In preferred embodiments, the subject drug screening assays are carried out in a manner designed to detect selectivity for specific NF-AT paralogs. For instance, in certain preferred embodiments, the assays are set up to detect NF-AT antagonists which are selective for NF-AT<sub>c3</sub> 10 and/or NF-AT<sub>c4</sub>, but not NF-AT<sub>c1</sub> or NF-AT<sub>c2</sub>. For instance, the NF-AT antagonist can be selected so as to have an ED50 for inhibition of NF-AT<sub>c3</sub> or NF-AT<sub>c4</sub> in vivo of at least one, and more preferably, two, three, four and even five orders of magnitude less than its ED50 for inhibition of NF-AT<sub>c1</sub> or NF-AT<sub>c2</sub> activity.

Such selectivity can take advantage of differences amongst two or more NF-AT paralogs 15 in specificity for DNA recognition elements, in the protein-protein interactions, and/or post-translational modification of NF-AT proteins. Thus, any of the assays described herein can be run, e.g., side-by-side, using two or more different NF-AT paralogs, and compounds identified not only on the basis of their ability to affect the biological activity of an NF-AT protein, but also on their ability to do so in a manner which is selective as between the NF-AT paralogs used in the assay.

20 In this regard, the present invention provides assays for identifying agents which are either agonists or antagonists of the normal cellular function of the subject NF-AT proteins, or of the role of those proteins in the pathogenesis of normal or abnormal cell function, such as muscle cell hypertrophy and disorders related thereto. In one embodiment, the assay evaluates the ability of a compound to modulate binding of an NF-AT protein with other proteins, DNA or RNA. In 25 other embodiments, the assay detects compounds which inhibit or potentiate the post-translation modification of an NF-AT protein, such as phosphorylation and/or changes in folding of the protein. Still other embodiments detect changes in the cellular localization of an NF-AT protein. Compounds identified by the present assay can be used, for example, in the treatment of diseases or conditions caused or contributed to by cell hypertrophy, in particular muscle cell hypertrophy. 30 For example, compounds of the invention can be used for treating congestive heart disease.

Agents to be tested for their ability to act as agonists or antagonists of an NF-AT protein can be produced, for example, by bacteria, yeast or other organisms (e.g. natural products), produced chemically (e.g. small molecules, including peptidomimetics), or produced recombinantly. In a preferred embodiment, the test agent is a small organic molecule having a 35 molecular weight of less than about 2,000 daltons. A high speed screen for agents that bind directly to the molecular regulator may employ immobilized or "tagged" combinatorial libraries

(or libraries which otherwise readily deconvoluted).

Agents that are identified as active in the drug screening assay are candidates to be tested for their capacity to block development of hypertrophy of cells and/or their capacity to reduce hypertrophy in a cell. As described below, these agents would be useful for treating or preventing 5 cardiac conditions including cardiac infarction and congestive heart disease.

A variety of assay formats will suffice and, in light of the present disclosure, those not expressly described herein will nevertheless be comprehended by one of ordinary skill in the art. For instance, the assay can be generated in many different formats, and include assays based on 10 cell-free systems, e.g. purified proteins or cell lysates, as well as cell-based assays which utilize intact cells. Simple binding assays can also be used to detect agents which, such as those which detect compounds able to potentiate or disrupt protein-protein or protein-DNA interaction involving an NF-AT protein.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in 15 a given period of time. Assays of the present invention which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins or with lysates, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored 20 in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with other proteins or changes in enzymatic properties of the molecular target.

Accordingly, in an exemplary screening assay of the present invention, a reaction mixture is generated to include an NF-AT protein, test compound(s), and a "target molecule", e.g., a 25 protein or nucleic acid which interacts with the NF-AT protein. Detection and quantification of interaction of the NF-AT protein with the target molecule provides a means for determining a compound's efficacy at inhibiting or potentiating interaction between the NF-AT protein and the target molecule. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay 30 can also be performed to provide a baseline for comparison. In the control assay, interaction of the NF-AT protein and target molecule is quantitated in the absence of the test compound.

Interaction between an NF-AT protein and an NF-AT binding partner may be detected by a variety of techniques. Modulation of the formation of complexes can be quantitated using, for example, detectably labeled proteins such as radiolabeled, fluorescently labeled, or enzymatically 35 labeled NF-AT proteins or NF-AT binding partners, by immunoassay, or by chromatographic detection.

Typically, it will be desirable to immobilize either NF-AT or its binding partner to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of NF-AT to an NF-AT binding partner, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/NF-AT (GST/NF-AT) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the NF-AT binding partner, e.g. an  $^{35}\text{S}$ -labeled NF-AT binding partner, and the test compound, and the mixture incubated under conditions conducive to complex formation, e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound label, and the matrix immobilized and radiolabel determined directly (e.g. beads placed in scintilant), or in the supernatant after the complexes are subsequently dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of NF-AT protein or NF-AT binding partner found in the bead fraction quantitated from the gel using standard electrophoretic techniques such as described in the appended examples.

Other techniques for immobilizing proteins on matrices are also available for use in the subject assay. For instance, either NF-AT or its cognate binding partner can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated NF-AT molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with NF-AT can be derivatized to the wells of the plate, and NF-AT trapped in the wells by antibody conjugation. As above, preparations of an NF-AT binding protein and a test compound are incubated in the NF-AT presenting wells of the plate, and the amount of complex trapped in the well can be quantitated. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the NF-AT binding partner, or which are reactive with NF-AT protein and compete with the binding partner; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the binding partner, either intrinsic or extrinsic activity. In the instance of the latter, the enzyme can be chemically conjugated or provided as a fusion protein with the NF-AT binding partner. To illustrate, the NF-AT binding partner can be chemically cross-linked or genetically fused with horseradish peroxidase, and the amount of polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme,

e.g. 3,3'-diamino-benzidine terahydrochloride or 4-chloro-1-naphthol. Likewise, a fusion protein comprising the polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) J Biol Chem 249:7130).

5 For processes which rely on immunodetection for quantitating one of the proteins trapped in the complex, antibodies against the protein, such as anti-NF-AT antibodies, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes, in addition to the NF-AT sequence, a second polypeptide for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion 10 proteins described above can also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include myc-epitopes (e.g., see Ellison et al. (1991) J Biol Chem 266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZ-Z-protein A system (Pharmacia, NJ).

15 An interaction between molecules, in particular between NF-AT and an NF-AT binding partner, can also be identified by using real-time BIA (Biomolecular Interaction Analysis, Pharmacia Biosensor AB) which detects surface plasmon resonance (SPR), an optical phenomenon. Detection depends on changes in the mass concentration of macromolecules at the biospecific interface, and does not require any labeling of interactants. In one embodiment, a 20 library of test compounds can be immobilized on a sensor surface, e.g., which forms one wall of a micro-flow cell. A solution containing the NF-AT protein, functional fragment thereof, NF-AT analog or NF-AT binding partner is then flown continuously over the sensor surface. A change in the resonance angle as shown on a signal recording, indicates that an interaction has occurred. This technique is further described, e.g., in BIATechnology Handbook by Pharmacia.

25 The above-described screening assays can generally be performed as follows. In this description, one component is NF-AT or a portion thereof and the other component is generally termed NF-AT binding partner, which can be, e.g., an NF-AT molecule or portion thereof, a kinase, or a phosphatase such as calcineurin. Thus, an exemplary screening assay of the present invention includes the steps of (a) forming a reaction mixture including: (i) an NF-AT polypeptide, 30 (ii) an NF-AT binding partner, and (iii) a test compound; and (b) detecting interaction of the NF-AT and the NF-AT binding protein. The reaction mixture can be a cell-free protein preparation, e.g., a reconstituted protein mixture or a cell lysate, or it can be a recombinant cell including a heterologous nucleic acid recombinantly expressing the NF-AT protein. For instance, the NF-AT polypeptide and NF-AT binding partner can be produced recombinantly, purified from a source, 35 e.g., a cell extract, or chemically synthesized, as described herein. A statistically significant change (increase or inhibition) in the interaction of the NF-AT and NF-AT binding protein in the

presence of the test compound, relative to the interaction in the absence of the test compound, indicates a potential agonist (mimetic or potentiator) or antagonist (inhibitor) of NF-AT bioactivity for the test compound. The compounds of this assay can be contacted simultaneously. Alternatively, an NF-AT protein can first be contacted with a test compound for an appropriate 5 amount of time, following which the NF-AT binding partner is added to the reaction mixture. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, isolated and purified NF-AT 10 polypeptide or binding partner is added to a composition containing the NF-AT binding partner or NF-AT polypeptide, and the formation of a complex is quantitated in the absence of the test compound.

The invention also provides screening assays for identifying compounds which modulate phosphorylation or dephosphorylation of an NF-AT molecule. In this regard, an NF-AT antagonist, at least with respect to treatment of cardiac hypertrophy, is an agent which either inhibits 15 dephosphorylation of an NF-AT protein, or potentiates phosphorylation. In certain embodiments of the assay, it may be desirable to directly detect changes in phosphorylation of an NF-AT polypeptides.

In one embodiment, the assay is an *in vitro* assay. In one embodiment, the assay comprises 20 contacting a non phosphorylated, or partially phosphorylated NF-AT polypeptide with a cell extract, or with one or more purified kinases, such as GSK-3 and PKA, and other necessary components of an *in vitro* kinase assay, including a source of phosphate and with or without a test compound and under conditions under which phosphorylation of NF-AT occurs. The comparison of the state of phosphorylation of NF-AT in the presence and in the absence of a test compound 25 will indicate whether the test compound decreases or inhibits, or alternatively increases or stimulates, the phosphorylation of NF-AT. The kinase assay and preparation of a cellular extract can be performed as described in the Examples. When the NF-AT polypeptide is partially phosphorylated prior to use in the kinase assay, this can be achieved, e.g., by prior incubation of a non-phosphorylated NF-AT with PKA. An NF-AT phosphorylated with PKA can be used as a component of an assay for identifying compounds which inhibit phosphorylation by GSK-3, since 30 GSK-3 phosphorylates peptides which have been phosphorylated by PKA. Non phosphorylated or partially phosphorylated NF-AT can also be obtained from cells containing active, e.g., nuclear NF-AT. Thus, NF-AT substrate for use in this assay can be obtained from or consist in a nuclear extract of activated T cell.

In another embodiment, the kinase assay is an *in vivo* kinase assay. The assay can 35 comprise incubating a cell expressing non phosphorylated or partially phosphorylated NF-AT, e.g., an activated T cell, with a test compound and comparing the state of phosphorylation of NF-AT

in the presence and in the absence of the test compound. A variation in the state of phosphorylation will indicate that the test compound is capable of modulation phosphorylation of NF-AT. The state of phosphorylation of NF-AT can be determined by, e.g., by performing the incubation of the cells in the presence of labeled, e.g., radioactive, phosphate (e.g., ATP), and 5 determining the amount of label present in an immunoprecipitate with an NF-AT specific antibody. Alternatively, the state of phosphorylation can be performed by Western blot analysis, optionally coupled with immunoprecipitations.

In another embodiment, the invention provides screening assays for identifying compounds which modulate dephosphorylation of NF-AT, such as inhibitors of calcineurin-mediated 10 dephosphorylation of an NF-AT proteins. In one embodiment, the assay comprises incubating a phosphorylated NF-AT polypeptide with a cell extract or with one or more phosphatases, e.g., calcineurin, in conditions under which the NF-AT polypeptide is phosphatased, and a test compound. NF-AT can be phosphorylated *in vitro* with PKA and optionally GSK-3, or NF-AT can be phosphorylated with a cell extract. NF-AT can also be isolated from or present in a cell 15 extract. The comparison of the state of phosphorylation of NF-AT after a phosphatase reaction in the presence and in the absence of a test compound will indicate whether the test compound is capable of modulating dephosphorylation of NF-AT. A higher level of phosphorylated NF-AT in the presence of the test compound relative to the level of phosphorylation in the absence of the test compound indicates that the compound is an inhibitor of NF-AT dephosphorylation. A lower 20 level indicates that the test compound is a stimulator of dephosphorylation. The state of phosphorylation of NF-AT can be determined as described above.

In yet another embodiment, the drug screening assay is derived to include a whole cell expressing an NF-AT protein. The ability of a test agent to alter the activity of the NF-AT protein can be detected by analysis of the recombinant cell. For example, agonists and antagonists of the 25 NF-AT protein biological activity can be detected by scoring for alterations in hypertrophy of the cell. General techniques for detecting such changes are well known, and are further described herein. For the cell-based assays, the recombinant cell is preferably a mammalian cell, e.g., a human cell. In a preferred embodiment, the cell is a muscle cell, most preferably a myocyte.

In addition to morphological studies, change(s) in the level of an intracellular second 30 messenger responsive to activities dependent on the NF-AT protein can be detected. For example, in various embodiments the assay may assess the ability of test agent to cause changes in phosphorylation patterns of NF-AT or expression of genes whose transcription is dependent on NF-AT. By detecting changes in intracellular signals, such as alterations in second messengers or gene expression, candidate agonists and antagonists to NF-AT protein-dependent signaling can be 35 identified.

By selecting transcriptional regulatory sequences from target genes, e.g., that are

responsible for the up- or down-regulation of these genes having NF-AT dependent transcriptional control elements, and operatively linking such promoters to a reporter gene, the present invention provides a transcription based assay which is sensitive to the ability of a specific test compound to influence signaling pathways dependent on the NF-AT protein.

5 In an exemplary embodiment, the subject assay comprises detecting, in a cell-based assay, change(s) in the level of expression of a gene controlled by a transcriptional regulatory sequence responsive to signaling by an NF-AT protein. Reporter gene based assays of this invention measure the end stage of the above described cascade of events, e.g., transcriptional modulation. Accordingly, in practicing one embodiment of the assay, a reporter gene construct is inserted into 10 the reagent cell in order to generate a detection signal dependent on signaling by the NF-AT protein. Expression of the reporter gene, thus, provides a valuable screening tool for the development of compounds that act as agonists or antagonists of NF-AT protein-dependent signalling.

15 In practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on second messengers generated by the NF-AT protein. Typically, the reporter gene construct will include a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to signal transduction from the NF-AT protein, with the level of expression of the reporter gene providing the detection signal. The amount of transcription from the reporter gene may be measured using any method 20 known to those of skill in the art to be suitable. For example, mRNA expression from the reporter gene may be detected using RNase protection or RNA-based PCR, or the protein product of the reporter gene may be identified by a characteristic stain or an intrinsic activity. The amount of expression from the reporter gene is then compared to the amount of expression in either the same cell in the absence of the test compound or it may be compared with the amount of transcription 25 in a substantially identical cell that lacks the target receptor protein. Any statistically or otherwise significant difference in the amount of transcription indicates that the test compound has in some manner altered the inductive activity of the NF-AT protein.

30 As described in further detail below, in preferred embodiments the gene product of the reporter is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detection signal based on color, fluorescence, or luminescence. In other preferred embodiments, the reporter or marker gene provides a selective growth advantage, e.g., the reporter gene may enhance cell viability, relieve a cell nutritional requirement, and/or provide resistance to a drug. Many reporter genes are known to those of skill in the art and others may be identified or synthesized by methods known 35 to those of skill in the art. A reporter gene includes any gene that expresses a detectable gene product, which may be RNA or protein.

Preferred reporter genes are those that are readily detectable. The reporter gene may also be included in the construct in the form of a fusion gene with a gene that includes desired transcriptional regulatory sequences or exhibits other desirable properties. Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek 5 (1979), *Nature* 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al. (1987), *Mol. Cell. Biol.* 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), *PNAS* 1: 4154-4158; Baldwin et al. (1984), *Biochemistry* 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) *Eur. J. Biochem.* 182: 231-238, Hall et al. (1983) *J. Mol. Appl. Gen.* 2: 101), human placental secreted alkaline phosphatase 10 (Cullen and Malim (1992) *Methods in Enzymol.* 216:362-368).

In still another embodiment of a drug screening, a two hybrid assay can be generated with an NF-AT protein and target molecule. Drug dependent inhibition or potentiation of the interaction can be scored. The two hybrid assay formats described in the art can be readily adapted for such drug screening embodiments. See, for example, U.S. Pat. Nos. 5,283,317, 5,580,736 and 15 5,695,941; Zervos et al. (1993) *Cell* 72:223-232; Madura et al. (1993) *J Biol Chem* 268:12046-12054; Bartel et al. (1993) *Biotechniques* 14:920-924; and Iwabuchi et al. (1993) *Oncogene* 8:1693-1696).

The identification of compounds which modulate dephosphorylation of NF-AT can also be identified in cell-based assays. For example, a cell containing phosphorylated NF-AT is 20 incubated in the presence or absence of a test compound and the state of phosphorylation of NF-AT is determined as described above. For instance, the ability of compounds to modulate NF-AT phosphorylation/de phosphorylation could be screened using colony immunoblotting (Lyons and Nelson (1984) *PNAS* 81:7426-7430) using antibodies against phosphorylated residues. Reagents for performing such assays are further described herein.

25 In yet another embodiment, the invention provides a cell-based screening assay for identifying compounds which modulate nuclear translocation of NF-AT comprising incubating or treating a cell with or without a test compound and determining the localization of NF-AT in the cell, i.e., whether NF-AT is present in the cytoplasm and/or in the nucleus of the cell. In one embodiment, a cell containing NF-AT in the cytoplasm, e.g., a resting T cell or a Jurkat cell, is 30 incubated with a test compound and the cellular localization of NF-AT is determined. The presence of NF-AT in the nucleus indicates that the test compound stimulates the translocation of NF-AT from the cytoplasm to the nucleus. In another embodiment, a cell containing NF-AT in the cytoplasm is incubated with a test compound and an agent which activates NF-AT, i.e., an agent which stimulates its translocation to the nucleus and the cellular localization of NF-AT is 35 determined. If more NF-AT is localized in the cytoplasm of the cell incubated with the test compound relative to a cell that was not treated with the test compound, the test compound inhibits

the translocation of NF-AT from the cytoplasm to the nucleus and is thus an NF-AT inhibitor. Alternatively, if more NF-AT is present in the nucleus of the cell incubated with the test compound, relative to the cell that was not incubated with the test compound, the test compound is a stimulator or activator of NF-AT.

5 The invention further provides screening assays for identifying compounds which modulate the activity of NF-AT. The screening assays can be *in vivo* or *in vitro* and can be cell based or based on a cell free format. In a preferred embodiment, the assays allow the identification of compounds which modulate NF-AT translocation across the nuclear membrane. In an even more preferred embodiment, the screening assay comprises contacting an NF-AT NLS with an NF-AT 10 molecule or portion thereof which is sufficient for binding to the NLS and with a test compound or a library of test compounds. In one embodiment, the NLS comprises the amino acid sequence CNKRKYSLN (SEQ ID NO: 53) (N-terminal NLS). In another embodiment, the NLS comprises the amino acid sequence GKRKK/R (SEQ ID NO: 67) (C-terminal NLS). The other component of the screening assay can be a peptide comprising the SRR, SP1, SP2, and/or SP3 of an NF-AT 15 molecule. In a preferred embodiment, the screening assay comprises an N-terminal NLS and a peptide comprising the SRR region, which is phosphorylated.

In another embodiment, the screening assay comprises a C-terminal NLS and a peptide comprising SP1, SP2 and/or SP3 regions which are phosphorylated.

20 In another embodiment of the screening assay, one component of the assay is an NF-AT polypeptide or portion thereof sufficient for binding to calcineurin and the other component is calcineurin or a portion thereof sufficient for binding to NF-AT. A portion of NF-AT can be an N-terminal portion, e.g., amino acids 1-418 of SEQ ID NO: 38. Thus, in one embodiment, the screening assay comprises contacting an NF-AT polypeptide with calcineurin and a test compound in conditions under which, but for the presence of the test compound, the NF-AT polypeptide are 25 capable of interacting. The comparison of the binding of the two components of the assay in the presence and in the absence of a test compound will indicate whether the test compound inhibits, or alternatively stimulates, the interaction between NF-AT and calcineurin.

30 In another embodiment, the screening assay comprises incubating a cell containing nuclear NF-AT, e.g., an activated T cell such as a T cell treated with ionomycin, is contacted with a test compound and the cellular localization of NF-AT is determined. If more NF-AT is localized in the nucleus in the cell treated with the test compound versus the cell that was not treated with the test compound, the test compound is a compound which is capable of maintaining NF-AT in an activated state, i.e., in the nucleus. If more NF-AT is localized in the cytoplasm in the cell treated 35 with the test compound relative to the cell that was not treated with the test compound, the test

compound is capable of deactivating NF-AT, i.e., to stimulate its translocation to the cytoplasm.

The cellular localization of an NF-AT molecule can be determined, by, e.g., detecting its localization within a cell using an antibody or other agent capable of specifically interacting with NF-AT. For example, NF-AT can be detected by immunfluorescence, such as described in the 5 Examples. In another embodiment, the cell can be transfected with a nucleic acid encoding an NF-AT polypeptide that is fused to a tag or marker, that can be detected. For example, the cell can be made to express an NF-AT polypeptide that is fused to a myc tag and the NF-AT fusion polypeptide can then be detected with an antibody binding specifically with a myc tag.

10 Other agents which are capable of modulating the hypertrophic state of cells include nucleic acids which compete away the binding of NF-AT proteins to their DNA binding site, e.g., "decoy" nucleic acids. Preferred nucleic acids comprise a DNA binding site of NF-AT. These reagents can be inhibit binding of only a single NF-AT polypeptide, or alternatively, reagents can be designed for inhibiting binding of most or all of the different NF-AT family members to their 15 DNA binding sites.

In addition to small molecules which may be identified, e.g., by the drug screening assays described above, other agents capable of modulating cell hypertrophy may include peptide domains (fragments) of the NF-AT protein, as well as mutants of the molecular regulators. A "mutant" as used herein refers to a peptide having an amino acid sequence which differs from that 20 of the naturally occurring peptide or protein by at least one amino acid. Mutants may have the same biological and immunological activity as the naturally occurring protein. However, the biological or immunological activity of mutants may differ or be lacking. For example, a protein mutant may act as an agonist, antagonist (competitive or non-competitive), or partial agonist of the function of the naturally occurring protein.

25 For example, homologs of the NF-AT proteins (both agonist and antagonist forms) can be generated using, for example, alanine scanning mutagenesis and the like (Ruf et al. (1994) Biochemistry 33:1565-1572; Wang et al. (1994) J. Biol. Chem. 269:3095-3099; Balint et al. (1993) Gene 137:109-118; Grodberg et al. (1993) Eur. J. Biochem. 218:597-601; Nagashima et al. (1993) J. Biol. Chem. 268:2888-2892; Lowman et al. (1991) Biochemistry 30:10832-10838; 30 and Cunningham et al. (1989) Science 244:1081-1085), by linker scanning mutagenesis (Gustin et al. (1993) Virology 193:653-660; Brown et al. (1992) Mol. Cell Biol. 12:2644-2652; McKnight et al. (1982) Science 232:316); by saturation mutagenesis (Meyers et al. (1986) Science 232:613); by PCR mutagenesis (Leung et al. (1989) Method Cell Mol Biol 1:11-19); or by random mutagenesis (Miller et al. (1992) A Short Course in Bacterial Genetics, CSHL Press, Cold Spring 35 Harbor, N.Y.; and Greener et al. (1994) Strategies in Mol Biol 7:32-34). Linker scanning mutagenesis, particularly in a combinatorial setting, is an attractive method for identifying

truncated (such as constitutively active or dominant negative) forms of an NF-AT protein.

The invention also contemplates the reduction of the subject NF-AT protein to generate mimetics, e.g. peptide or non-peptide agents, which are able interfere with, or mimic, the effect of the authentic NF-AT protein on the hypertrophic state of cells, such as muscle cells. Such 5 peptidomimetics can act as drugs for the modulation of cell hypertrophy.

Peptidomimetics are commonly understood in the pharmaceutical industry to include non-peptide drugs having properties analogous to those of the mimicked peptide. The principles and practices of peptidomimetic design are known in the art and are described, for example, in Fauchere, Adv. Drug Res. 15:29 (1986); and Evans et al., J. Med. Chem. 30:1229 (1987).

10 Peptidomimetics which bear structural similarity to therapeutically useful peptides may be used to produce an equivalent therapeutic or prophylactic effect. Typically, such peptidomimetics have one or more peptide linkages optionally replaced by a linkage which may convert desirable properties such as resistance to chemical breakdown-*in vivo*. These linkages may include -CH<sub>2</sub>NH-, -CH<sub>2</sub>S-, -CH<sub>2</sub>-CH<sub>2</sub>-, -CH=CH-, -COCH<sub>2</sub>-, -CH(OH)CH<sub>2</sub>-, and -CH<sub>2</sub>SO-.

15 Peptidomimetics may exhibit enhanced pharmacological properties (biological half life, absorption rates, etc.), different specificity, increased stability, production economies, lessened antigenicity and the like which makes their use as therapeutics particularly desirable.

Such mutagenic techniques as described above are also particularly useful for mapping the determinants of an NF-AT protein which participate in protein-protein interactions involved in, 20 for example, binding to a leucine-zipper containing protein. To illustrate, the critical residues of an NF-AT protein which are involved in molecular recognition of other cellular proteins (or nucleic acid) can be determined and used to generate peptidomimetics which maintain at least a portion of that binding activity. By employing, for example, scanning mutagenesis to map the amino acid residues involved in binding, peptidomimetic compounds (e.g. diazepine or 25 isoquinoline derivatives) can be generated which mimic those residues in binding to the kinase. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), 30 substituted gamma lactam rings (Garvey et al. in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) J. Med. Chem. 29:295; and Ewenson et al. in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, Ill., 1985),  $\beta$ -turn dipeptide cores (Nagai et al. (1985) Tetrahedron Lett. 26:647; and Sato et al. (1986) J. Chem. Soc. Perkin Trans. 1:1231), and  $\beta$ -aminoalcohols (Gordon et al. (1985) Biochem Biophys Res Commun. 126:419; and Dann et al. (1986) Biochem Biophys Res Commun. 134:71).

NF-AT<sub>c</sub> polypeptides, especially those portions which form direct contacts in NF-AT complexes, can be used for rational drug design of candidate NF-AT-modulating agents (e.g., antineoplastics and immunomodulators). The substantially purified NF-AT<sub>c</sub> and the identification of NF-AT<sub>c</sub> as capable of forming intermolecular associations, e.g., with AP-1 and with DNA, as well as forming intermolecular associations, as provided herein, permits production of substantially pure NF-AT polypeptide complexes and computational models which can be used for protein X-ray crystallography or other structure analysis methods, such as the DOCK program (Kuntz et al. (1982) *J. Mol. Biol.* 161: 269; Kuntz ID (1992) *Science* 257: 1078) and variants thereof. Potential therapeutic drugs may be designed rationally on the basis of structural information thus provided. In one embodiment, such drugs are designed to prevent formation of a NF-AT<sub>c</sub> polypeptide: AP-1 polypeptide complex. In another embodiment, such drugs are designed to prevent the formation of intramolecular interactions in NF-AT. Thus, the present invention may be used to design drugs, including drugs with a capacity to inhibit binding of NF-AT<sub>c</sub> to form an NF-AT complex.

15

*Cardiac hypertrophy models*

Compounds identified by the above-described methods can be further evaluated as agents for treating cardiac hypertrophy and the like using any of a number of cardiac hypertrophy models. Methods for inducing cardiac hypertrophy: induction by a variety of humoral factors, including 20 angiotensin II, phenylephrine (PE), and endothelin-1 (ET-1) (Karliner et al. (1990) *Experientia* 46:81, Sadoshima et al. (1993) *Circ. Res.* 73:424, Leite et al. (1994) *Am. J. Physiol.* 267: H2193).

Compounds or polypeptides or genes encoding polypeptides which are capable of inducing 25 cardiac hypertrophy are referred to herein as "hypertrophy inducing" compounds, polypeptides or genes, respectively. Several investigators have shown that endothelin-1, which is known to be produced in endothelial cells, induces hypertrophy of cardiac myocytes in vitro. Shubeita et al. (1990) *J. Biol. Chem.*, 265:20555-20562; Ito et al., (1991) *Circ. Res.* 69: 209-215; Suzuki et al., (1991) *J. Cardiovasc. Pharmacol.*, 17 Suppl 7: S182-S186. See also U.S. Pat. No. 5,344,644. Yet another hypertrophy inducing factor is LIF (U.S. Pat. No. 5837241).

In vitro and in vivo methods for determining the presence of muscle cell hypertrophy, e.g., 30 ventricular muscle cell hypertrophy, are known. In vitro assays for muscle cell hypertrophy include those methods described herein, e.g., increased cell size and increased expression on atrial natriuretic factor (ANF). Changes in cell size are used in a scoring system to determine the extent of hypertrophy. These changes can be viewed with an inverted phase microscope, and the degree of hypertrophy scored with an arbitrary scale of 7 to 0, with 7 being fully hypertrophied cells, and 35 3 being non-stimulated cells. The 3 and 7 states may be seen in Simpson et al., (1982) *Circulation Res.* 51:787-801, FIG. 2, A and B, respectively. The correlation between hypertrophy score and

cell surface area ( $\mu\text{m}^2$ ) has been determined to be linear (correlation coefficient = 0.99). In phenylephrine-induced hypertrophy, non-exposed (normal) cells have a hypertrophy score of 3 and a surface area/cell of  $581 \mu\text{m}^2$  and fully hypertrophied cells have a hypertrophy score of 7 and a surface area/cell of  $1811 \mu\text{m}^2$ , or approximately 200% of normal. Cells with a hypertrophy score of 4 have a surface area/cell of  $771 \mu\text{m}^2$ , or approximately 30% greater size than non-exposed cells; cells with a hypertrophy score of 5 have a surface area/cell of  $1109 \mu\text{m}^2$ , or approximately 90% greater size than non-exposed cells; and cells with a hypertrophy score of 6 have a surface area/cell of  $1366 \mu\text{m}^2$ , or approximately 135% greater size than non-exposed cells. The presence of ventricular muscle cell hypertrophy preferably includes cells exhibiting an increased size of about 15% (hypertrophy score 3.5) or more. Inducers of hypertrophy vary in their ability to induce a maximal hypertrophic response as scored by the above-described assay. For example, the maximal increase in cell size induced by endothelin is approximately a hypertrophy score of 5.

A hypertrophic assay can be performed as follows. First, a myocyte cell suspension can be prepared as described in Chien et al., (1985) *J. Clin. Invest.*, 75: 1770-1780 and Iwaki et al., *supra*. Ventricles from the hearts of 1-2 day Sprague-Dawley rat pups are removed and trisected. The minced ventricles are digested with a series of sequential collagenase treatments. Purification of the resulting single-cell suspension on a discontinuous Percoll gradient results in a suspension of 95% pure myocytes.

The culture of myocytes can then be plated as described in Long et al., *supra*. This method includes preplating the cell suspension for 30 min. in MEM/5% calf serum. The unattached myocytes can then be plated in serum-free MEM supplemented with insulin, transferrin, BrdU, and bovine serum albumin in 35-mm tissue-culture dishes at a density of  $7.5 \times 10^4$  cells per mL. The culture of myocytes can also be plated in D-MEM/199/5% horse serum/5% fetal calf serum in 10-cm tissue-culture dishes at  $3 \times 10^5$  cells per mL. After 24 hr in culture the cells are washed and incubated in serum-free D-MEM/199.

Yet another method for culturing myocytes to increase testing capacity with a miniaturized assay, is as follows. The wells of 96-well tissue-culture plates are precoated with D-MEM/F12/4% fetal calf serum for 8 hr at 37 °C. This medium is removed and the cell suspension is plated in the inner 60 wells at  $7.5 \times 10^4$  cells per mL in D-MEM/F-12 supplemented with insulin, transferrin, and aprotinin. The medium typically also contains an antibiotic such as penicillin/streptomycin and glutamine. This medium allows these cells to survive at this low plating density without serum. Test substances, e.g., NF-AT antagonists are added directly into the wells after the cells have been in culture for 24 hours.

After stimulation with  $\alpha$ -adrenergic agonists or endothelin, neonatal rat myocardial cells in culture display several features of the *in vivo* cardiac muscle cell hypertrophy seen in congestive

heart failure, including an increase in cell size and an increase in the assembly of an individual contractile protein into organized contractile units. Chien et al., *FASEB J.*, *supra*. These changes can be viewed with an inverted phase microscope and the degree of hypertrophy scored with an arbitrary scale of 7 to 0, with 7 being fully hypertrophied cells and 3 being non-stimulated cells.

5 The 3 and 7 states may be seen in Simpson et al., (1982) *Circulation Research*, 51: 787-801. To facilitate the microscopic readout of the 96-well cultures and to generate a permanent record, the myocytes are fixed and stained after the appropriate testing period with crystal violet stain in methanol. Crystal violet is a commonly used protein stain for cultured cells. Additionally, an aliquot can be taken from the 96-well plates and monitored for the expression of protein markers 10 of the response such as release of ANF or ANP (as described, e.g., in U.S. Pat. No. 5534615).

At a high cell density, myocytes may also begin to self-induce hypertrophy.

Thus, in one embodiment, a method for screening for NF-AT antagonists which inhibit hypertrophy comprises the following steps:

15 (a) plating 96-well plates with a suspension of myocytes at a cell density of about  $7.5 \times 10^4$  cells per mL in D-MEM/F-12 medium supplemented with at least insulin, transferrin and aprotinin;

(b) culturing the cells in the presence of a hypertrophy inducing factor, e.g., LIF or endothelin;

(c) adding a test substance to be assayed (an NF-AT antagonist);

20 (d) culturing the cells with the test substance; and

(e) measuring for hypertrophy.

The medium can be supplemented with additional elements such as EGF that ensure a longer viability of the cells, but such supplements are not essential. D-MEM/F-12 medium is available from Gibco BRL, Gaithersburg, Md.

25 The preferred hypertrophy assay comprises:

(a) pre-coating the wells of 96-well tissue culture plates with a medium containing calf serum, preferably D-MEM/F-12 medium containing 4% fetal calf serum, wherein preferably the wells are incubated with the medium for about eight hours at about 37 °C;

(b) removing the medium;

30 (c) plating a suspension of myocytes in the inner 60 wells at  $7.5 \times 10^4$  cells per mL in D-MEM/F-12 medium supplemented with insulin, transferrin and aprotinin;

(d) culturing the myocytes for at least 24 hours in the presence of a hypertrophy inducing factor, e.g., LIF or endothelin;

(e) adding the test substance;

35 (f) culturing the cells with the test substance (preferably for about 24-72 hours, more preferably for about 48 hours); and

(g) evaluating hypertrophy, preferably after crystal violet stain, e.g., by microscopic examination.

Preferably the medium used in step (c) is a serum-free medium also containing penicillin/streptomycin (pen/strep) and glutamine. Most preferably, the medium contains 100 mL  
5 D-MEM/F-12, 100 mu L transferring (10 mg/mL), 20 mu L insulin (5 mg/mL), 50 mu L aprotinin (2 mg/mL), 1 mL pen/strep (JRH Biosciences No. 59602-77P), and 1 mL L-glutamine (200 mM).

The assay capacity of 1000 single samples a week coupled with the small sample size requirement of 100 mu L or less has enabled an expression cloning and protein purification that would have been impossible to accomplish using the current methods available.

10 Another method for assaying hypertrophy involves measuring for atrial natriuretic peptide (ANP), a marker for myocyte hypertrophy, release by means of an assay that determines the competition for binding of  $^{125}$ I-rat ANP for a rat ANP receptor A-IgG fusion protein. The method suitable for use is similar to that used for determining GP130 using a CD4-IgG fusion protein described by Chamow et al., *Biochemistry*, 29: 9885-9891 (1990). Cultures of myocytes  
15 and hypertrophy induction and assaying are further described in U.S. Pat. No. 5837241.

Alternatively, a compound of the invention for inhibiting cardiac hypertrophy can be identified by screening for compounds which inhibit NF-ATc activity. Preferably, the compound inhibits NF-ATc4 activity, but does not inhibit the activity of one or more protein members of the NF-AT family, i.e., NF-ATc1, NF-ATc2, or NF-ATc3 and splice variants thereof. Alternatively,  
20 the antagonists for use in the invention could inhibit the activity of more than one NF-AT polypeptide, but not of all of them. For example, an antagonist for use in the invention could be an NF-ATc4 and NF-ATc3 antagonist, but not an NF-ATc1 or NF-ATc2 antagonist. Compounds that are antagonists of only certain NF-AT polypeptides, but not of others can be developed based on the differences between the NF-AT family members. For example, antagonists specific for certain  
25 NF-AT polypeptides can be compounds which inhibit interaction of an NF-AT polypeptide with a specific DNA binding sequence. Since the NF-AT polypeptides do not all bind the same NF-AT binding sequence, antagonists that are specific for certain NF-AT polypeptides can be developed. Alternatively, antagonists that are specific for certain NF-AT polypeptides can be compounds which are based on differences in the NF-AT sequences. For example, antisense compounds can  
30 be used for specifically inhibiting the production of only some NF-AT polypeptide and not that of others. Similarly, ribozymes can be designed to selectively destroy certain members of the NF-AT family, without affecting others.

Alternatively, compounds which are capable of inhibiting all of the NF-AT family members can also be designed, based on the significant homologies between these proteins and  
35 also between the genes encoding them.

Agents which can be used to prevent or treat cardiac hypertrophy can also be identified in

animal models. Animal models can be animals which develop cardiac hypertrophy and animal models in which these animals are crossed with an NF-AT knock-out mouse of the invention.

In an illustrative embodiment, the animal model is a transgenic mouse having a constitutively active NF-AT pathway. Such mice include those having a constitutive calcineuring 5 or a constitutive NF-AT, e.g., NF-ATc4, such as those described in Olsen et al., Cell 93: 215 (1998). These mice can be bred with the NF-ATc4 knock-out mice of the invention. Thus, as opposed to transgenic mice having a constitutively active NF-ATc4 signaling pathway, and which express wild-type NF-ATc4, mice which have a constitutively active NF-ATc4 pathway, but which do not express wild-type NF-ATc4 will not develop cardiac hypertrophy.

10 Other animal models which can be used alone, e.g., in screening assays, or which can be crossed with the NF-ATc4 knock-out mice to obtain animal models, include mice which are transgenic for a hypertrophy inducing gene, such as those described herein. Another animal model of cardiac hypertrophy includes the pressure-overload mouse model wherein the pulmonary artery is constricted, resulting in right ventricular failure. A retroviral murine model of ventricular 15 dysfunction can also be used. Other animal models include MLC-ras mice and aortic banding of heterozygous IGF-I-deficient mice. Additionally, transgenic mice that harbor a muscle actin promoter-IGF-I fusion gene display cardiac and skeletal muscle hypertrophy, without evidence of myopathy or heart failure. IGF-I-gene-targeted mice display defects in cardiac myogenesis (as well as skeletal), including markedly decreased expression of ventricular muscle contractile 20 protein genes. Another useful animal models include the RXR alpha mutant mouse model (Sucov et al. (1994) Genes Dev. 8:1997-1018) and RXR alpha -/-embryo model (Dyson et al. (1995) Proc. Natl. Acad. Sci. (In Press)). These genetically-based animal models display important features of ventricular chamber dysmorphogenesis.

An NF-AT antagonist can also tested in a post-myocardial infarction rat model, which is 25 predictive of human congestive heart failure in producing ANF. A detailed description of the procedure for obtaining such rats is described in U.S. Pat. No. 5767155. Briefly, myocardial infarction is produced in male Sprague-Dawley (Charles River Breeding Laboratories, Inc., eight weeks of age) by left coronary arterial ligation as described by Greenen et al. (1987) J. Appl. Physiol. 93:92-96 and Buttrick et al. (1991) Am. J. Physiol. 260:11473-11479. Four to six weeks 30 after ligation, myocardial infarction can develop into heart failure in rats. The development of infarcts can be monitored by performing electrocardiograms. The congestive heart failure in this model reasonably mimics congestive heart failure in most human patients.

A person of skill in the art will recognize that any of the above described screening assays 35 can easily be adapted for use in screening libraries of compounds. The compounds identified using any of the screening assays of the invention are also within the scope of the invention, as well as pharmaceutical compositions and kits comprising such.

In another aspect, the present invention provides pharmaceutically acceptable compositions which comprise a therapeutically-effective amount of one or more of the compounds described above, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. As described in detail below, the pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes for application to the tongue; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; or (4) intravaginally or intravectally, for example, as a pessary, cream or foam.

The phrase "therapeutically-effective amount" as used herein means that amount of a compound, material, or composition comprising a peptide or peptidomimetic of the present invention which is effective for producing some desired therapeutic effect by inhibiting an NF-AT dependent signaling pathway in at least a sub-population of cells in an animal and thereby blocking the biological consequences of that pathway in the treated cells, at a reasonable benefit/risk ratio applicable to any medical treatment.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically-acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject peptidomimetic agent from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-

free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

5     J. Exemplary Uses of the invention

The invention provides methods for modulating the activity of NF-AT in a cell. In one embodiment, the activity of NF-AT is modulated by modulating its interaction with another molecule, such as another protein or a nucleic acid. In particular, the activity of NF-AT can be modulated by modulating the interaction between NF-ATc and AP-1 or other basic domain/leucine zipper proteins. In another embodiment, the interaction of NF-ATc with DNA, i.e., the NF-ATc binding site, can be modulated.

In a preferred embodiment, the method modulates the translocation of NF-AT through the nuclear membrane. For example, certain methods of the invention stimulate or inhibit translocation of NF-AT proteins from the cytoplasm to the nucleus. Other methods of the invention stimulate or inhibit translocation of NF-AT molecules from the nucleus to the cytoplasm. For example, the translocation of NF-AT from the cytoplasm into the nucleus of a cell can be stimulated or induced by introducing into the cell a compound which inhibits the interaction of at least one NLS with another part of the NF-AT molecule, thereby unshielding at least one NF-AT NLS, allowing the NF-AT molecule to translocate into the nucleus. A compound which inhibits the interaction between an NLS and another portion of the NF-AT protein can be a small molecule, a peptide, peptidomimetic, a nucleic acid or derivatives thereof. A preferred compound is peptide or peptidomimetic comprising an NF-AT NLS, or homolog thereof, which is capable of forming an intramolecular association with another portion of the NF-AT molecule, such as with one or more of the repeats, e.g., SRR, SP1, SP2, or SP3. Such compounds are further described herein. Another preferred compound is a nucleic acid which encodes such a peptide. Accordingly, the nucleic acid is introduced into a cell expressing NF-AT and expressed in the cell. In another embodiment, the compound is a small molecule, which can be isolated as described herein by screening libraries of small molecules. The compound is preferably small and able to cross the cytoplasmic membrane.

30     In another embodiment, the invention provides a method for inhibiting the translocation of NF-AT from the cytoplasmic to the nucleus, thereby inhibiting NF-AT dependent biologic activities. This can be achieved, e.g., by stabilizing the intramolecular association of at least one NLS with another portion of an NF-AT molecule. Methods may include introducing into a cell a compound which stabilizes the interaction of an NLS with at least one portion of NF-AT selected from the group consisting of SRR, SP1, SP2 and SP3. The compound is preferably a small molecule, which can be obtained as described herein.

In yet another embodiment, the invention provides a method for stimulating the translocation of NF-AT from the nucleus to the cytoplasm of a cell, thereby blocking activation of NF-AT dependent biological activities. This can be achieved, e.g., by shielding one or more of the NLSs in NF-AT molecules. In an illustrative embodiment, an NLS sequence in an NF-AT molecule in a cell is shielded by introducing into the cell a compound which interacts with the NLS sequence. A preferred compound is a peptide or peptidomimetic, e.g., a peptide comprising an amino acid sequence selected from the group consisting of the SRR, SP1, SP2, or SP3 sequence of NF-AT. In a preferred embodiment, two compounds are introduced into a cell, i.e., a first compound which interacts with the NLS KRK (amino acids 265-267 of SEQ ID NO: 38) and a second compound which interacts with the NLS KRKK/R (SEQ ID NO: 66) (amino acids 682-685 of SEQ ID NO: 38). For example, a peptide comprising the amino acid sequence of SRR and a peptide comprising the amino acid sequence of at least one of SP1, SP2 and SP3 can be administered to a cell.

Furthermore, also within the scope of the invention are methods for inhibiting the translocation of NF-AT molecules from the nucleus to the cytoplasm of a cell, thereby maintaining or prolonging NF-AT dependent biological activities. Such methods can comprise introducing into the nucleus of a cell comprising NF-AT molecules in the nucleus a compound which prevents one or more of the NLSs to form an intramolecular association. This can be achieved, e.g., by introducing into the cell a compound which interacts with a portion of NF-AT which is capable of interacting with an NLS. For example, the compound can be an NLS peptide or derivative thereof, which is capable of binding to a portion of an NF-AT molecule, e.g., an SRR, SP1, SP2 and/or SP3 repeat. Based at least on the sequence homologies between the NF-ATc family members, a single peptide could interact with at least two NF-ATc family members. Other compounds that can be used for this purpose include nucleic acids encoding such peptides and small molecules.

Other methods for modulating translocation of an NF-ATc polypeptide comprise modulating phosphorylation of NF-ATc, such as the phosphorylation of serines located in the region from about amino acid 1 to about amino acid 418 of SEQ ID NO: 38, or even more preferably from about amino acid 170 to about amino acid 301 of SEQ ID NO: 38. Even more preferably, the method comprises phosphorylation of serines located in SRR, SP1, SP2, and/or SP3, and/or in regions located between these repeats.

Phosphorylation of NF-ATc polypeptides can be modulated by a variety of methods. In one embodiment, phosphorylation is modulated by modulating the activity of a kinase which phosphorylates NF-ATc, such as PKA, GSK-3 $\alpha$  and GSK-3 $\beta$ . Another kinase whose activity can be phosphorylated include JNK (jun kinase), e.g., JNK-1 or JNK-2. The activity of a kinase can be modulated by modulating the protein level of the kinase. For example, increasing the activity

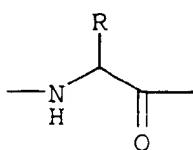
of a kinase can be accomplished by increasing the endogenous protein level of the kinase, such as by increasing transcription of the kinase. Alternatively, the activity of a kinase can be increased by introducing a kinase into a cell, such as by introducing a nucleic acid encoding the kinase. In fact, as shown herein, overexpression of GSK-3 in T cells inhibited translocation of NF-AT to the 5 nucleus, and increased nuclear export of NT-ATc. Stimulation of translocation of NF-AT from the cytoplasm to the nucleus can be achieved by inhibiting the activity of a kinase which phosphorylates NF-ATc, such as by inhibiting transcription or translation of the kinase, e.g., by using antisense technology. The activity of the kinase can also be inhibited by introducing into the cell an agent which inhibits its activity, such as an NF-AT peptide, capable of binding to the 10 kinase.

In another embodiment, phosphorylation is modulated by modulating the activity of a phosphatase, such as calcineurin. This can be achieved by modulating its phosphorylation capacity, by, e.g., contacting it with an NF-AT peptide with which calcineurin is capable of interacting. Alternatively, the level of calcineurin in a cell can be modulated, such as by 15 modulating its expression or by introducing exogenous calcineurin in the cell or by introducing antisense nucleic acids inhibiting calcineurin mRNA translation.

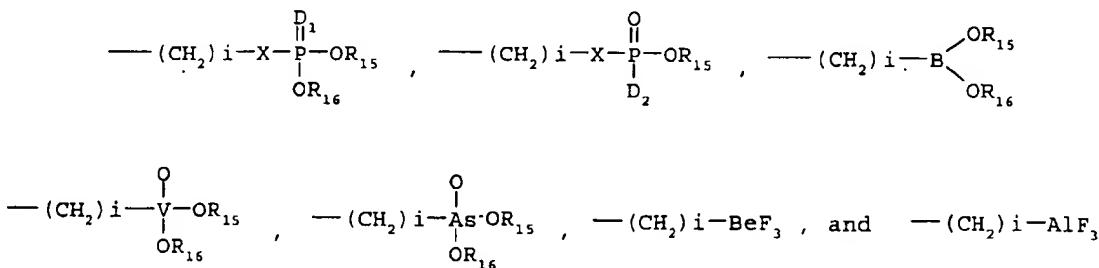
In still another embodiment, the present invention provides compounds which inhibit either the phosphorylation of an NF-AT protein by a glucan synthase kinase like GSK-3, or dephosphorylation of NF-AT by a phosphatase such as calcineurin. In this regard, the application 20 provides drug screening assays based on monitoring the rate of phosphorylation of NF-AT, e.g., a particularly significant residues, by GSK-3 in the presence of absence of a test compound. Likewise, the application describes drug screening assays based on monitoring the rate of dephosphorylation of NF-AT, e.g., a particularly significant residues, by calcineurin in the presence of absence of a test compound.

25 On salient feature to our discovery that GSK-3 is a specific phosphatase for NF-AT proteins, and the elucidation of particular residues (e.g., the NLS sites) as substrates for GSK-3, the present invention also provides peptide and peptidimimetic inhibitors of GSK-3. Such inhibitors can correspond to 4 or more residues of NF-AT, and can be in the range of 4-25, more preferably 4-15 and even more preferably 4-10. The inhibitors preferably have Ki's for inhibition 30 of GSK-3 phosphorylation of an NF-AT of 1 $\mu$ M or less, more preferably of 100mM or less, and even more preferably of 1nM or less.

35 Preferably, the peptide or peptidimimetic inhibitor of GSK-3 includes a phosphoserine residue, or, even more preferably, an analog thereof. The phosphoserine moiety can be represented by the general formula



where R is selected from a group consisting of



5

wherein i is zero or an integer in the range of 1 to 6; X is absent or represents O, S, or N; D<sub>1</sub> represents O or S; D<sub>2</sub> represents N<sub>3</sub>, SH<sub>2</sub>, NH<sub>2</sub>, or NO<sub>2</sub>; and R<sub>15</sub> and R<sub>16</sub> each independently represent hydrogen, a lower alkyl, or a pharmaceutically acceptable salt, or R<sub>15</sub> and R<sub>16</sub> taken together with the O-P-O, O-B-O, O-V-O or O-As-O atoms to which they are attached complete a heterocyclic ring having from 5 to 8 atoms in the ring structure. In a preferred embodiment, the phosphoserine is a non-hydrolyzable phosphoserine analog.

For illustrative purposes, peptide analogs of the present invention can be generated using benzodiazepines, substituted gamma lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p123), C-7 mimics (Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p. 105), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985),  $\beta$ -turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans 1*:1231),  $\beta$ -aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71), diaminoketones (Natarajan et al. (1984) *Biochem Biophys Res Commun* 124:141), and methyleneamino-modified (Roark et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p134). Also, see generally, Session III: Analytic and synthetic methods, in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988)

In other exemplary embodiments, the peptidomimetic can be derived as a retro-inverso analog of a peptide sequence, such as that described by the Sisto et al. U.S. Patent 4,522,752, as

a retro-enatio analog of the peptide, as a trans-olefin derivatives such as can be synthesized according to the method of Y.K. Shue et al. (1987) *Tetrahedron Letters* 28:3225, or as a phosphonate derivative, such as can be adapted from such synthesis schemes as, Loots et al. in *Peptides: Chemistry and Biology*, (Escom Science Publishers, Leiden, 1988, p. 118); Petrillo et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium, Pierce Chemical Co. Rockland, IL, 1985).

The invention also provides methods for providing activation of NF-AT dependent biological activities by introducing into a cell a constitutively active NF-AT, e.g., an NF-AT polypeptide in which the NLS cannot form intramolecular interactions with other parts of the protein, thereby resulting in an NF-AT protein which constitutively translocates to the nucleus. Such NF-AT proteins can, e.g., have substitutions of serines in the SRR, SP1, SP2, and/or SP3 regions of the NF-AT protein, such that at least one NLS of the NF-AT is "unshielded". Thus, the activity of a constitutively active NF-AT protein can be modulated by modulating the expression of a gene encoding the protein. For example, the gene encoding the constitutively active NF-AT can be placed under the control, e.g., transcriptional control, of an inducible regulatory element.

Another constitutively active NF-AT polypeptide that can be used according to the methods of the invention comprises one or more additional NLS. These can be NF-AT NLS or heterologous NLSs, such as the SV40 large T antigen NLS.

Alternatively, NF-AT dependent biological activities can be modulated as follows. An NF-AT polypeptide that is mutated so that it is constitutively active, is fused to a ligand binding domain and expressed in a cell which further expresses a cytoplasmic retention domain fused to a ligand binding domain, such that in the presence of a dimerizer, the two fusion proteins are cross-linked and the NF-AT protein is retained in the cytoplasm. Translocation of the NF-AT protein into the nucleus can be induced by the absence of, or removal of, the dimerizer.

In yet another embodiment, the invention provides a method for regulating the expression of NF-AT by regulating the expression of a dominant negative mutant of NF-AT. In one embodiment, a dominant negative mutant is an NF-AT protein that is incapable of translocating to the nucleus, e.g., an NF-AT polypeptide in which at least one, but preferably two NLSs are inactivated, e.g., by mutation. Such mutant NF-AT polypeptides are still capable of interacting with calcineurin, and will thereby compete away calcineurin from endogenous NF-AT molecules.

In yet another embodiment, the invention provides a method for regulating translocation of a heterologous polypeptide across the nuclear membrane that is not dependent on intracellular calcium. Accordingly, a heterologous polypeptide is fused an NLS of NF-AT, e.g., the C-terminal NLS, and to an NF-AT portion selected from the group consisting of SRR, SP1, SP2, and SP3. Preferably, the NF-AT portion is an SRR. The cellular localization of a heterologous polypeptide fused to these portions of NF-AT in a cell will depend on the presence of calcium. Thus, in

normal conditions, it is expected that the protein will be located in the cytoplasm of the cell and that, in the presence of a calcium ionophore, the polypeptide will translocate to the nucleus. Alternatively, a heterologous polypeptide can be fused to an NLS only and its intracellular location modulated by addition to the cell of a compound, e.g., a peptide that interacts with the NLS.

5 Moreover, GSK-3 has been shown to be involved in dorsal-ventral pattern formation in *Xenopus* (He et al., *Nature*. 374, 617 (1995)) and in segment polarity determination in *Drosophila*, where it was discovered as *zest white 3* or *shaggy* (Bourouis et al., *EMBO J.* 9, 2877 (1990); Siegfried et al., *Nature* 345, 825 (1990)). GSK-3 is a negative regulator of the wnt signaling pathway and it has been shown that loss of function and dominant negative mutations in GSK-3  
10 beta lead to activation of the wnt pathway in *Drosophila* and *Xenopus*. Furthermore, the Wingless signaling pathway to GSK-3 is conserved in mammals (Cook et al., *EMBO J.* 15, 4526 (1996); Stambolic et al., *Curr. Biol.* 6, 1664 (1996)) and the wnt signaling pathway plays a central role in the development of invertebrates and vertebrates. Thus, it is likely that the Wingless signaling pathway controls the nuclear export of NF-AT family members in the tissues where these genes  
15 are coexpressed. Accordingly, the invention also provides methods for regulating NF-AT translocation in a cell, comprising contacting the cell with a compound which modulates the Wingless signaling pathway. For example, translocation of NF-AT from the cytoplasm to the nucleus can be stimulated with activators of the wnt signaling pathway. In yet another embodiment, the invention provides a method for modulating the Wingless signaling pathway,  
20 comprising, e.g., modulating the activity of GSK-3. In one embodiment, the activity of GSK-3 is inhibited with an NF-AT peptide capable of binding to GSK-3, thereby activating the Wnt signaling pathway..

25 The methods of the invention can be used for treating or preventing in a subject a disease or condition that is associated with abnormal or aberrant T cell activation. Diseases or conditions involving, i.e., caused by or contributed to by, an excessive T cell activation include cancers, such as leukemias, inflammation, or autoimmune diseases. Alternatively, the methods of the invention can also be used to immunosuppress a subject, e.g., a recipient of a graft such as an organ or bone marrow transplant patient. Diseases or conditions involving an abnormally low T cell activation  
30 include immunosuppressed states, e.g., AIDS or conditions in which a subject has an infection. Thus, for example, a subject having a viral or bacterial infection can be treated by administering to the subject a compound which activates NF-ATc thereby activating the T cells of the subject and stimulating the immune system of the subject for fighting against the infection. For example, one can administer, either locally or systemically to the subject a pharmaceutically effective  
35 amount of a compound which increases NF-ATc localization in the nucleus, such as a compound which inhibits the intramolecular interaction between an NLS in NF-ATc and another portion of

the NF-AT molecule. On the contrary, where a subject has an autoimmune disease, it is desirable to inhibit or reduce T cell activation. Thus, in this situation, one can administer to the subject a pharmaceutically effective amount of a compound which increases NF-ATc localization in the cytoplasm, e.g., a compound which shields the NLS of NF-ATc. Alternatively, a compound that 5 activates GSK-3 and/or PKA activity and NF-AT phosphorylation can be administered to the subject.

Furthermore, since the Wingless signaling pathway involves GSK-3, the invention provides methods for treating diseases or disorders associated with the Wingless signaling pathway, such as cancer, e.g., breast cancer. For example, a disease can be treated or prevented 10 by administering to a subject having such a disease a compound which is capable of inhibiting GSK-3, e.g., an NF-AT peptide capable of binding to GSK-3.

In one embodiment, the NF-AT antagonists described herein can be used to inhibit unwanted vascular tissue proliferation, e.g., either in tissue culture or *in vivo*. For example, the 15 subject antagonists can be used to inhibit growth of cardiac myocytes and/or nonmyocytes (fibroblast). In other embodiments, the subject method can be used to inhibit arteriolar smooth muscle proliferation.

For example, NF-AT antagonists, particularly antagonists of NF-ATc3 and/or NF-ATc4, can be used to inhibit cardiac and/or vascular hypertrophy, e.g., as part of treatment or prophylaxis 20 for cardiac injury. For example, the subject antagonists can be used to treat or prevent hypertrophic conditions following angioplasty, e.g., coronary angioplasty, treatment of restenosis and aortic stenosis, as well as part of a post-myocardial infarction regimen.

The subject antagonists can also be used as part of treatment for hypertension, especially 25 hypertensive heart disease.

In other embodiments, the NFAT antagonists of the present invention can be used as part of treatment for cardiomyopathy due to pathological stimuli, such as viral myocarditis.

In still other embodiments, the antagonists can be used as part of treatment to counter effect drugs which have an adverse side effect of promoting cardiac hypertrophy, such as thyroid 30 hormone treatment

Thus, in a preferred embodiment, the invention provides methods for preventing and 35 treating diseases and conditions in a mammal, such as a human, relating to NF-ATc mediated cellular hypertrophy, in particular, myocyte hypertrophy. In particular, the invention provides a method for treating a subject experiencing heart failure to prevent or lessen hypertrophy. In an illustrative embodiment, the method comprises administering chronically to a mammal in need of such treatment a therapeutically effective amount of an NF-AT antagonist, preferably an NF-ATc4 antagonist.

5        Optionally, the NF-ATc4 antagonist is chronically administered in combination with an effective amount of an antagonist to endothelin or LIF (see U.S. Pat. No. 5837241). Additional optional components include a cardiotrophin inhibitor such as a CT-1 antagonist, an ACE inhibitor, such as captopril, and/or human growth hormone and/or IGF-I in the case of congestive heart failure, or with another myocardiotrophic, anti-arrhythmic, or inotropic factor in the case of other types of heart failure or cardiac disorder, another anti-hypertrophic or myocardiotrophic factor in the case of other types of heart failure or cardiac disorder. Treatment of cardiac hypertrophy with agents is further described in U.S. Patent No. 5,573,762 by Ferarra et al.

10      ACE inhibitors which may be used as part of a conjoint therapeutic regimen are angiotensin-converting enzyme inhibiting drugs which prevent the conversion of angiotensin I to angiotensin II. The ACE inhibitors may be beneficial in congestive heart failure by reducing systemic vascular resistance and relieving circulatory congestion. ACE inhibitors include drugs designated by the trademarks Accupril Registered TM (quinapril), Altace Registered TM (ramipril), Capoten Registered TM (captopril), Lotensin Registered TM (benazepril), Monopril Registered TM (fosinopril), Prinivil Registered TM (lisinopril), Vasotec Registered TM (enalapril), and Zestril Registered TM (lisinopril).

20      The present invention can also be combined with the administration of drug therapies for the treatment of heart diseases such as hypertension. For example, an NF-AT antagonist can be administered with endothelin receptor antagonists, for example, an antibody to the endothelin receptor, and peptide or other small molecule antagonists; beta -adrenoceptor antagonists such as carvedilol; alpha 1-adrenoceptor antagonists; anti-oxidants; compounds having multiple activities (e.g., beta -blocker/ alpha -blocker/anti-oxidant); carvedilol-like compounds or combinations of compounds providing multiple functions found in carvedilol; growth hormone, etc.

25      **K.    Kits**

30      The compounds of the invention can be provided in the form of kits, for use in treating, preventing, or diagnosing diseases or conditions in which one desires to modulate the activity of T cells. For example, the invention provides kits for activating NF-ATc in a subject, comprising a compound which is capable of inhibiting intramolecular interaction of an NLS in NF-AT, or which is capable of inhibiting GSK-3 and/or PKA.

35      In a preferred embodiment of the invention, the kit of the invention provides reagents for determining the level of immunosuppression of a subject, such as a subject who is undergoing a treatment with an immunosuppressive drug. In one embodiment, the kit comprises a reagent for determining the cellular localization of NF-AT, such as an antibody that specifically binds to NF-AT. Other reagents that can be included in the kit are control reagents or standards, against which the results of the test can be compared. In some embodiments, the standard is a table or curve

indicating values in normal, e.g., non immunosuppressed, individuals. The kit may also contain other reagents, such as a secondary reagent, e.g., fluorescein labeled antibody and any buffer. In addition to monitoring the extent of immunosuppression of a subject, the kit of the invention can also be used to predict the sensitivity of a subject to a certain drug, e.g., an immunosuppressive drug.

5        **L. Methods for Forensic Identification**

The NF-AT<sub>c</sub> polynucleotide sequences of the present invention can be used for forensic identification of individual humans, such as for identification of decedents, determination of 10 paternity, criminal identification, and the like. For example but not limitation, a DNA sample can be obtained from a person or from a cellular sample (e.g., crime scene evidence such as blood, saliva, semen, and the like) and subjected to RFLP analysis, allele-specific PCR, or PCR cloning and sequencing of the amplification product to determine the structure of the NF-AT<sub>c</sub> gene region. On the basis of the NF-AT<sub>c</sub> gene structure, the individual from which the sample originated will 15 be identified with respect to his/her NF-AT<sub>c</sub> genotype. The NF-AT<sub>c</sub> genotype may be used alone or in conjunction with other genetic markers to conclusively identify an individual or to rule out the individual as a possible perpetrator.

In one embodiment, human genomic DNA samples from a population of individuals (typically at least 50 persons from various racial origins) are individually aliquoted into reaction 20 vessels (e.g., a well on a microtitre plate). Each aliquot is digested (incubated) with one or more restriction enzymes (e.g., EcoRI, HindIII, SmaI, BamHI, SalI, NotI, AccI, ApaI, BglII, XbaI, PstI) under suitable reaction conditions (e.g., see New England Biolabs 1992 catalog). Corresponding digestion products from each individual are loaded separately on an electrophoretic gel (typically agarose), electrophoresed, blotted to a membrane by Southern blotting, and hybridized with a 25 labeled NF-AT<sub>c</sub> probe (e.g., a full-length human NF-AT<sub>c</sub> cDNA sequence of Fig. 1). Restriction fragments (bands) which are polymorphic among members of the population are used as a basis to discriminate NF-AT<sub>c</sub> genotypes and thereby classify individuals on the basis of their NF-AT<sub>c</sub> genotype.

Similar categorization of NF-AT<sub>c</sub> genotypes may be performed by sequencing PCR 30 amplification products from a population of individuals and using sequence polymorphisms to identify alleles (genotypes), and thereby identify or classify individuals.

The present invention is further illustrated by the following examples which should not be construed as limiting in any way. The contents of all cited references (including literature 35 references, issued patents, published patent applications as cited throughout this application) are hereby expressly incorporated by reference. The practice of the present invention will employ,

unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2<sup>nd</sup> Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; Nucleic Acid Hybridization(B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

20            **Experimental Examples**

**Overview**

We have purified two related proteins encoded by separate genes that represent the preexisting or cytosolic components of NF-AT. Expression of a full length cDNA for one of these proteins, NF-AT<sub>c</sub>, activates the IL-2 promoter in non-T lymphocytes, while a dominant negative of NF-AT<sub>c</sub> specifically blocks activation of the IL-2 promoter in T lymphocytes, indicating that NF-AT<sub>c</sub> is required for IL-2 gene expression and is responsible for the restricted expression of IL-2. NF-AT<sub>c</sub> RNA expression is largely restricted to lymphoid tissues and is induced upon cell activation. The second protein, NF-AT<sub>p</sub>, is highly homologous to NF-AT<sub>c</sub> over a limited domain, but exhibits wider tissue distribution and is highly expressed in tissues characterized by Ca<sup>++</sup>-dependent regulation. Together these proteins are members of a new family of DNA binding proteins, which are distantly related to the Dorsal/Rel family (Nolan and Baltimore (1992) Current Biology, Ltd. 2: 211-220). Agents that increase intracellular Ca<sup>++</sup> or that activate protein kinase C independently produce alterations in the mobility of NF-AT<sub>c</sub>, indicating that distinct signaling pathways converge on NP-AT<sub>c</sub> to regulate its function.

35            Since our previous work indicated that the cytosolic component of NF-AT

was present at relatively low concentrations in human lymphoid cell lines (Northrop et al. (1993) *J. Biol. Chem.* **268**: 2917-2923), we chose to purify NF-AT<sub>c</sub> from bovine thymus. Amino acid sequence, obtained from 6 peptides, was used to isolate two overlapping human cDNA clones spanning 2742 nucleotides (Fig. 1). The cDNA encodes a protein of 716 amino acids with a predicted molecular weight of 77,870. An in-frame stop codon upstream from the initiator methionine indicates that the entire NF-AT<sub>c</sub> protein is encoded by this cDNA. A unique repeated sequence of 13 residues was also identified. The carboxy-terminal half of NF-AT<sub>c</sub> shows limited similarity to the DNA binding and dimerization regions of the Dorsal/Rel family of transcription factors (Fig. 4, for review, Nolan and Baltimore (1992) *Current Biology, Ltd.* **2**: 211-220) however, NF-AT<sub>c</sub> appears to be the most distantly related member of the group. There are a significant number of amino acid changes resulting in charge reversals between the Rel family members and NF-AT<sub>c</sub>, suggesting that charge might be conserved at these positions to maintain salt bridges. Six additional peptides obtained from the purified bovine protein are derived from the bovine homolog of NF-AT<sub>p</sub>, a cDNA fragment of which was reported by McCaffrey et al. (1993) *Science* **262**: 750-754). Comparison of NF-AT<sub>c</sub> and NF-AT<sub>p</sub> reveals that they are products of distinct genes with 73% amino acid identity in the Rel similarity region (Fig. 4), however, there is very little similarity outside this region. A murine cDNA for NF-AT<sub>c</sub> was isolated and the predicted protein was found to be 87% identical to human NF-AT<sub>c</sub>, and distinctly different from murine NF-AT<sub>p</sub>.

20

**Example 1: Determination of the nucleotide and amino acid sequence of human NF-AT<sub>c</sub> cDNA**

This example represents the isolation and purification of this novel human NF-AT protein, NF-AT<sub>c</sub>, the determination of the amino acid sequence of its fragments and the isolation and sequencing of the cDNA clone encoding this protein.

The protein was purified from bovine thymus glands obtained from newborn calves. Approximately 20 bovine thymuses were homogenized to make a cytosolic extract which was then subjected sequentially to 1) ammonium sulfate precipitation, 2) sulphopropyl Sepharose chromatography, 3) heparin agarose chromatography, 4) affinity chromatography using a multimerized binding site for NF-AT, with the sequence 5'-ACGCCCAAAGAGGAAAATTGTTCATACA-3' (SEQ ID NO: 73) coupled to sepharose CL4B, and 5) HPLC on a reverse phase C4 column. The resulting purified protein was subjected to cleavage with LysC/ArgC and fragments isolated by HPLC. The sequences of these individual

fragments were then determined by automated Edman degradation. Sequences obtained included: LRNSDIELRKGETDIGR (SEQ ID NO: 74) and LRNADIELR.(SEQ ID NO: 75). Degenerate oligos corresponding to GETDIG (SEQ ID NO: 76) (reverse primer) and RNADIE (SEQ ID NO: 77)(forward primer) were made. The degenerate oligo PCR primers had the following sequences:

5 A forward: (A/C)GIAA(C/T)GCIGA(C/T)AT(A/C/T)GA(A/G) (SEQ ID NO: 78)  
A reverse: ICC(A/G/T)AT(A/G)TCIGT(C/T)TCICC (SEQ ID NO: 79) .

10 To isolate the cDNA, oligonucleotide probes were made corresponding to the determined amino acid sequence and used as PCR primers to isolate a 45 base fragment from bovine cDNA prepared from the bovine thymus. The bovine PCR product comprised the nucleotide sequence CTG CGG AAA which encodes -L-R-K-. The same 45 bp fragment can be amplified from human and mouse sources.

15 This bovine PCR product was then used to screen a cDNA library of the human Jurkat T cell line. Clones were isolated at frequencies of about 1 in 100,000 to 1 in 200,000. A total of five human cDNA clones of various lengths were isolated. Two overlapping clones, one containing the 5' end and one containing the 3' end were ligated together using a unique EcoRI restriction site present in each clone, to produce a full-length cDNA which corresponded in length to the messenger RNA determined by Northern blotting.

20 The sequence of the NF-AT<sub>c</sub> cDNA was determined by the Sanger method and the complete nucleotide and predicted amino acid sequence is shown in Fig. 1. The initiator methionine indicated in Fig. 1 (boldface, indicated) was determined by fusing this reading frame to a glutathione transferase gene and transfecting the resultant clone into bacteria. The resultant clone produced a fusion protein of the proper molecular weight, indicating that the reading frame designated with the initiator methionine is indeed the correct reading frame. The position of the stop codon was determined by a similar procedure. In addition, the stop codon corresponds to the 25 reading frame for nine of the determined amino acid sequences.

30 The total NF-AT<sub>c</sub> protein structure was aligned against individual Rel proteins using a MacIntosh shareware program called DOTALIGN utilizing the alignment parameters of the FASTA programs. Significant homology was observed that corresponded to the Rel domains of these proteins. Enhanced amino acid residue alignment was done using ALIGN from the same suite of programs. Alignment of the Rel similarity regions of NF-AT<sub>c</sub> and NF-AT<sub>p</sub> was done by hand with no insertions necessary, The Miyata alphabet (Miyata et al. (1979) J. Mol. Evol. 12: 214-236) was used to determine similar residues. Fig. 4 shows results of such sequence alignments.

5           **Example 2: Expression of NF-AT<sub>c</sub> in T and non-T cells**

The cDNA shown in Fig. 1 was fused to the Hemophilus influenza hemagglutinin (HA) 12 amino acid epitope tag in the determined reading frame and operably linked to the SRα promoter in the vector pBJ5 (Lin et al, 1990, Science 249:677-679). The resultant construct was transiently transfected by electroporation into Jurkat human T lymphocytes, and into Cos fibroblast cells. Expression of the epitope-tagged NF-AT<sub>c</sub> protein was determined by Western blotting of whole cell extracts prepared from the transfected cells, using an antibody (12CA5, Berkeley Antibody Co., CA) that detects the HA epitope. Fig. 2 shows that NF-AT<sub>c</sub> cDNA construct is able to express a protein of approximately 120 kDa corresponding precisely in size to that of the purified protein, in both Jurkat T cells and Cos cells (see lanes 3 and 6 labeled NF-AT<sup>\*</sup>). Lane 2 shows as control, NF-AT without the epitope tag which cannot be detected in the Western blot).

15           **Example 3: Transfection of NF-ATc activates transcription in both Cos and Jurkat cells**

The NF-ATc cDNA was operably linked to a portion of the SV40 early gene promoter and the HIV transcription regulatory regions in the pBJ vector. This expression vector was co-transfected into Jurkat and Cos cells with either a) three copies of NF-AT binding site linked to and directing transcription of luciferase (results shown in Fig. 3A and 3B) the entire IL-2 enhancer/promoter directing transcription of luciferase (results shown in Fig. 3B). Cytosolic extracts were prepared and luciferase assays carried out by standard procedures (de Wet et al, 1987, Mol. Cell. Biol. 7:724-837).

20           The results demonstrate that in both Cos cells and Jurkat cells, overexpression of the NF-AT<sub>c</sub> protein dramatically enhances NF-AT-dependent transcription by 50-1000 fold (see Fig. 3A). In addition, overexpression of the NF-ATc protein in Cos cells activates the IL-2 promoter, which in the absence of NF-AT<sub>c</sub> cannot otherwise be activated (see Fig. 3B).

25           These results indicate that the cDNA clone encodes a functional NF-AT<sub>c</sub> protein and that NF-AT<sub>c</sub> is the protein which restricts expression of interleukin-2 to T cells.

30           **Example 4: NF-AT<sub>c</sub> mRNA and Protein Expression**

NF-AT<sub>c</sub> mRNA is absent in HeLa cells (Fig. 5, panel a, lane 7), a cell line incapable of IL-2 or NF-AT-dependent transcription, but is inducible in Jurkat cells (Fig. 5, panel a). This induction is sensitive to cyclosporin A, (CsA), indicating that NF-AT<sub>c</sub> may participate

in an auto-stimulatory loop as CsA has been shown to block its nuclear association (Flanagan et al. (1991) *Nature* **352**: 803-807). Two B cell lines, muscle tissue, Hep G2 cells and myeloid leukemia cells do not express NF-AT<sub>c</sub> mRNA (Fig. 5, panel b). These observations are consistent with the observed T cell-restricted pattern of IL-2 transcription and NF-AT activity. Previous 5 studies (Verweij et al. (1990) *J. Biol. Chem.* **265**: 15788-15795) revealed NF-AT-dependent transcription predominantly in spleen, thymus and skin of transgenic mice expressing an NF-AT-dependent reporter gene. Consistent with these observations, murine NF-AT<sub>c</sub> mRNA shows the same pattern of expression (Fig. 5 panel c). Small amounts of NF-AT<sub>c</sub> expression are seen in lung and heart, however, this may be due to contamination with circulating T cells. Murine NF-AT<sub>p</sub> mRNA, also assayed by quantitative ribonuclease protection, was found to be expressed at 10 approximately equal levels in brain, heart, thymus and spleen (Fig 5, panel c) . In contrast to NF-AT<sub>c</sub>, NF-AT<sub>p</sub> was not inducible by PMA and ionomycin (Fig 5, panel c).

METHODS. Specific human or mouse NF-AT<sub>c</sub> or mouse NF-AT<sub>p</sub> cDNA fragments were used as templates for the synthesis of RNA transcripts. Ribonuclease protection 15 was done according to Melton et al. (1984) *Nucl. Acids. Res.* **12**: 7035-7056) using 10  $\mu$ g of total RNA. Splenocytes and thymocytes were isolated and treated as described (Verweij et al. (1990) *J. Biol. Chem.* **265**: 15788-15795) before isolating RNA, otherwise whole tissue was used.

**Example 5: Functional Expression of NF-AT**

20 NF-AT luciferase and IL-2 luciferase have been described (Northrop et al. (1993) *J. Biol. Chem.* **268**: 2917-2923).  $\beta$ 28 luciferase was constructed by inserting a trimerized HNF-I recognition site ( $\beta$ 28) in place of the NF-AT recognition sites in NF-AT luciferase. The plasmid pSV2CAT (Gorman et al. (1982) *Mol. Cell. Biol.* **2**: 1044-1050) was used as an internal 25 control for transfection efficiency. Cells were transfected with 1.5 ug of luciferase reporter and 3 ug of expression construct as described. After 20 hours of growth, cells were stimulated for 8 hrs. with 20 ng/ml PMA plus or minus 2 uM ionomycin, and harvested for luciferase (de Wet et al. (1987) *Mol. Cell. Biol.* **7**: 725-737) and CAT assays (Gorman et al. (1982) *Mol. Cell. Biol.* **2**: 1044-1050).

30 Cos cells were transfected with epitope tagged NF-AT<sub>c</sub> as described. Cos cells, Jurkat cells, and murine thymocytes were stimulated for 3hr. with PMA and ionomycin. Hela cells were stimulated for 3hr with PMA alone and nuclear extracts prepared as described (Fiebing et al. (1990) *Genes & Dev.* **4**: 1823-1834). Cytosols were prepared from non-stimulated Cos cells. Gel mobility shifts were performed as previously described (Flanagan et al. (1991)

Nature 352: 803-807; Northrop et al. (1993) J. Biol. Chem. 268: 2917-2923). Antisera were raised in mice immunized with bacterially expressed glutathione S-transferase fusion proteins using the vector pGEX-3X (Pharmacia) and purified on glutathione agarose. Fusion proteins contained NF-AT<sub>c</sub> residues 12 to 143 (immune-1) and 12 to 699 (immune-2).

5 NF-AT<sub>c</sub>, expressed in non T cell lines specifically activated transcription from the NF-AT site and the IL-2 promoter, (Fig. 6 panel a (left), and Fig. 6 panel b ) . In transiently transfected Jurkat cells, overexpression of NF-AT<sub>c</sub> activated an NF-AT-dependent promoter but not an HNF-1 dependent promoter (Fig. 6 panel a (right)) or an AP-1-dependent promoter. Transfection of the NF-AT<sub>c</sub> cDNA gives rise to DNA binding activity that is indistinguishable from endogenous NF-AT (Fig. 6 panel c, lanes 1-4). Antibody directed against the HA epitope encoded by the transfected cDNA induces a supershift of the NF-AT complex indicating that NF-AT<sub>c</sub> participates in this activity. The nuclear NF-AT activity in transfected Cos cells comigrates with, and has the same binding specificity as, the native nuclear complex in T-cells (Fig. 6 panel c, lanes 4-11). cytosolic extracts from NF-AT<sub>c</sub>, transfected Cos cells can 10 reconstitute NF-AT DNA binding activity when mixed with Hela nuclear extract (Fig. 6 panel c, lanes 12-16) as do cytosolic extracts from T-cells (Flanagan et al. (1991) Nature 352: 803-807; Northrop et al. (1993) J. Biol. Chem. 268: 2917-2923). Antisera raised against bacterially expressed fragments of NF-AT<sub>c</sub> that have no similarity to NF-AT<sub>p</sub> are able to induce a supershift of the endogenous NF-AT complex, but not the AP-1 complex, from Jurkat cells or thymocytes 15 (immune-1 and immune-2 respectively, Fig. 6 panel d). Immune-2 antisera reduced the DNA-protein complex produced using murine thymic nuclear extracts significantly, consistent with the relatively equal representation of NF-AT<sub>c</sub>, and NF-AT<sub>p</sub> peptides in the purified protein from bovine thymus.

20

25 **Example 6: NF-AT<sub>c</sub> dominant negative mutant assayed in transient transfection assays**

A dominant negative NF-AT<sub>c</sub>, prepared after extensive deletion analysis of the cDNA, indicated that the amino terminal domain would block NF-AT-dependent function without affecting binding. This region of the cDNA is not found in NF-AT<sub>p</sub> and hence can be used 30 to assess the contribution of NF-AT<sub>c</sub> to the activation of the IL-2 gene. The dominant negative NF-AT<sub>c</sub> used consists of a carboxy terminal truncation of the epitope tagged NF-AT<sub>c</sub> expression plasmid (*supra*) extending to the PvuII site at amino acid 463. Transfection of this dominant negative resulted in more than 90% inhibition of IL-2 promoter function as well as transcription

directed by the NF-AT site (Fig 7). This effect was highly specific since transcription directed by the AP-1 site or the RSV promoter and enhancer were relatively unaffected (Fig 7). These results strongly indicate that NF-AT<sub>c</sub> contributes substantially to IL-2 gene expression in T cells.

5 Dominant-negative NF-AT<sub>c</sub> polypeptides or peptidomimetics thereof can be used as pharmaceutical antagonists of NF-AT-mediated activation of T cells. In one variation, such drugs can be used as commercial research reagents for laboratory testing and analysis of T cell activation and the like, among many other uses (e.g., immunosuppressant).

**Example 7: Post-Translational Modification of NF-AT<sub>c</sub>**

10 Post-translational modification of NF-AT<sub>c</sub> was investigated in cells treated with agents that activate PKC or increase intracellular Ca<sup>++</sup>. Cells were transfected with NF-AT<sub>c</sub> as described in Fig. 2 and stimulated as shown for 2 hrs plus or minus 100ng/ml CsA. Whole cell lysates were analyzed by western blotting as in Fig. 2. The bulk of NF-AT<sub>c</sub> in cells treated with ionomycin migrates faster than that in non-treated cells and this mobility shift is inhibited by CsA (Fig. 8, lanes 1, 3-4). This is consistent with a dephosphorylation event, possibly by direct action of calcineurin (Clipstone and Crabtree (1992) *Nature* 357: 695-697), however, any of a large number of processes could produce the observed mobility changes. There is evidence that NF-AT<sub>p</sub> is a substrate for calcineurin, however, the mobility shifts produced by phosphatase treatment of NF-AT<sub>p</sub> or NF-AT<sub>c</sub> are far greater than those observed in Figure 8. These observations indicate 15 that NF-AT<sub>c</sub> is not a direct substrate of calcineurin. PMA treatment produces a slower migrating NF-AT<sub>c</sub> (Fig. 8, lane 2); therefore, PKC-activated pathways likely contribute to NF-AT activity 20 by modification of NF-AT<sub>c</sub> in addition to activation of the nuclear component.

**Example 8: Calcineurin is the rate-limited for NF-ATc nuclear entry**

25 Most tissues express one of the NF-AT family members. A variety of cell types, including lymphocytes and fibroblasts, support the Ca<sup>2+</sup>-dependant nuclear localization of transfected as well as endogenous NF-ATc family members (Shibasaki et al., *Nature*, 382: 370-373). To develop an accurate assay for NF-AT translocation, NF-ATc was expressed in COS cells which, unlike lymphocytes, have abundant cytoplasm and hence allow easier assessment of 30 cytoplasmic and nuclear localization.

COS-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM; Sigma) with 10% fetal calf serum (FCS), 100 µg/ml of penicillin G, 100 µg/ml of streptomycin, and 10 mM HEPES (pH 7.4) at 37°C in 5% CO<sub>2</sub>. Cells were transfected by electroporation with

1 µg of SH160c Ho et al. (1995) *J. Biol. Chem.* 270:19898, which encodes the FLAG epitope tag inserted at an *Xba*I site immediately 5' to the second codon of the human NF-ATc1 cDNA in the pBJ5 vector (*Nature*, 369:497-502)), plated on glass coverslips, and stimulated 18-14 hr post-transfection in fresh media or fresh media supplemented with ionomycin (2 µM final) plus 10 mM (final) CaCl<sub>2</sub> for various amounts of time at 37°C. Ionomycin was obtained from Calbiochem and dissolved in DMSO. Cells were also treated with FK506 at 2 ng/ml plus ionomycin and CaCl<sub>2</sub> or with ionomycin plus 2.5 mM EGTA for 60 min. FK506 was added at 2 ng/ml 15 min prior to addition of calcium and ionomycin. FK506 was obtained from Fukisawa (Chicago, IL) and dissolved in ethanol. Efficient nuclear translocation of NF-ATc in COS cells requires both ionomycin and the elevation of extracellular calcium. The reason for this requirement for Ca<sup>2+</sup> may be that ionomycin stimulation of COS cells does not result in an intracellular Ca<sup>2+</sup> level as high as stimulated lymphocytes. Cells were then stained with the anti-FLAG antibody as follows. Cells adhering to coverslips were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. The FLAG epitope was detected by incubating with 1 µg/ml of anti-FLAG M2 antibody (Eastman Kodak Co.). The monoclonal antibody was detected by incubation with biotin-conjugated anti-mouse IgG (Caltag), followed by streptavidin-FITC and DAPI (Molecular Probes). Fluorescence was visualized with a Zeiss Axiophot fluorescence microscope. Fluorescent cells in which the nucleus and plasma membrane could be identified were scored as containing predominantly cytoplasmic staining, predominantly nuclear staining, or both cytoplasmic and nuclear staining. At least 100 cells were scored on each coverslip. Cells undergoing mitosis or with multiple nuclei were excluded. For all deletion constructs, the subcellular localization was confirmed using a confocal imaging fluorescence microscope.

As shown in the diagram in Figure 9B, as with NF-ATc3(4) (Shibasaki et al., *Nature*, 382: 370-373), the amino terminus of NF-ATc1 was sufficient for Ca<sup>2+</sup>-regulated nuclear import that was blocked by FK506. Furthermore, transfected NF-ATc1 moved into the nucleus within 5-15 min after ionomycin treatment.

Exit of NF-ATc from the nucleus was determined by stimulating transfected cells with I+Ca<sup>2+</sup> for 1 hr and then replacing the medium with medium containing FK506. Slides were fixed at various time points, and the percentage of cells with cytoplasmic NF-ATc was determined. Cells expressing NF-ATc in the cytoplasm and those expressing NF-ATc in both cytoplasm and nucleus were added and divided by the total number of analyzed expressing cells. The results, which are shown in Figure 9C indicate that NF-AT moved out of the nucleus and into the cytoplasm within 30 min of FK506 addition. These translocations occurred even if

protein synthesis was inhibited. The full-length protein behaved similar to a fusion of the amino-terminal 418 amino acid with green fluorescent protein (NF-AT(C<sub>Δ</sub>418)-GFP) (Fig. 9A). pSH160cΔ418-GFP construct was made by fusing the *Bam*HI-*Not*I fragment encoding GFP from pEGFP-1 (Clonetech) following the *Pvu*II site at codon 418 of SH160c and was detected using its autofluorescence.

This time course of nuclear localization is consistent with that observed in murine lymphocytes activated by antigen presentation (Timmerman et al., *Nature*, 383: 837-840) and indicates that COS cells can support physiologic translocation of NF-ATc. As with NF-ATc3[4] (Shibasaki et al., *Nature*, 382: 370-373), overexpression of calcineurin enhanced the movement 10 of NF-AT into the nucleus of COS cells (Fig. 9A), indicating that calcineurin is rate limiting for the movement of NF-ATc proteins into the nucleus.

**Example 9: Addition of heterologous nuclear localization sequences to NF-ATc results in Ca<sup>2+</sup>-independent, FK506-resistant nuclear import**

The observation that overexpressed NF-ATc is cytoplasmic in Jurkat T lymphocytes and COS cells suggests that there is not an easily saturated cytoplasmic anchoring protein necessary to retain NF-ATc in the cytoplasm. Transfection of the NF-ATc1 expression construct over a 200-fold range of DNA concentration did not result in higher levels of constitutive nuclear localization. If NF-ATc was localized by a cytoplasmic anchoring partner, 15 the addition of a fully active nuclear localization sequence (NLS) to NF-ATc should not overcome the cytoplasmic retention NF-ATc. Accordingly, NF-ATc1 expression constructs with zero, one, or two copies of either the SV40 large T-antigen NLS encoded between the FLAG epitope and the second amino acid of NF-ATc1, or one or two copies of a mutant form of the NLS (NLS-T). The constructs bearing the SV40 NLS and mutant (NLS-T) were created by 20 insertion of synthetic oligo-nucleotides at the *Xba*I site of pSH160c (Ho et al. (1995) *J. Biol. Chem.* 270:19898, which encodes the FLAG epitope tag inserted at an *Xba*I site immediately 5' to the second codon of the human NF0ATc1 cDNA in the pBJ5 vector (Northrop et al. (1994) *Nature*, 369:497-502)). The inserted NLS is CTAGTCCTAAGAAGAAGAGAAAGGTAT (SEQ ID NO: 80); the sequence of NLS-T is CTAGTCCTAAGACGAAGAGAAAGGTAT 25 (SEQ ID NO: 81) and substitutes a threonine for a lysine (*Cell*, 39:499-509). All point substitutions were created by sequential overlap extension PCR (*J. Biol. Chem.*, 270:19898-19900). Cells were then stained with an anti-FLAG antibody and the percentage of cells with nuclear fluorescence determined.

As shown in Figure 10, expression of NF-ATc1 with zero, one, or two copies of the SV40 large T-antigen NLS in COS cells results in a progressive increase in constitutive nuclear localization which was insensitive to FK506. In contrast, addition of the mutant NLS sequence, NLS-T (Kalderon et al. (1984) *Cell*, 39: 499-509), to NF-ATc1 resulted in substantially less nuclear entry. The low level of nuclear localizations resulting from inclusion of NLS-T may be attributable to slight activity of this mutant, which, like the wild-type sequence, is enhanced when present in multiple copies (Roberts et al. (1987) *Cell*, 50: 465-475). These results argue against a mechanism of cytoplasmic localization dependent on a dominantly acting cytoplasmic binding protein.

10

**Example 10: Two NLSs are each sufficient for NF-ATc nuclear translocation**

NF-ATc proteins contain four groups of clustered basic residues that are conserved among NF-ATc proteins which could possibly be NLSs (see Figure 11A). To determine whether these sequences have NLS activity, each of them was linked individually to the cytoplasmic exchange factor SOS.

The SOS expression constructs were based on the human SOS cDNA tagged at the carboxyl terminus by a HA epitope, pSOS-E (*Proc. Natl. Acad. Sci.* 92:9810-9814). PSOS-265 was created by insertion of an oligonucleotides encoding the sequence LECNKRKYSLNVD (SEQ ID NO: 82) at the unique *SaII* site between SOS and the HA epitope. An expression construct encoding SOS (SOS-E) was expressed and visualized with 12CA5 antibody. Constructs encoding SOS-E attached to residues 263-271 of NF-ATc (SOS-265) or attached to residues 681-685 of NF-ATc (SOS-682) were also detected with the 12CA5 antibody. Immunofluorescence was as described above, except that the HA epitope was detected by incubating with 1:2000 dilution of 12CA5 ascites.

25 The results, show that SOS-682, incorporating residues 682-685 of NF-ATc, and SOS-265, incorporating residues 265-267, are localized in the nucleus. Thus, these two conserved regions within NF-ATC are thus NLSs.

To determine whether these NLSs are required for nuclear import of NF-ATc, each sequence was mutated separately and in combination within the context of full-length NF-ATc1. A diagram of the mutations made in the NLS in the wild-type NF-ATc sequence is shown 30 in Figure 11B. The NLS at residues 265-268 was changed to QIL (construct m265). The NLS at residues 682-685 was changed to TRTG and the construct containing this mutation and m265 is referred to as m265 + 682. The mutant expression constructs were transfected in COS cells,

and the cells were stimulated with I+Ca<sup>++</sup> for 60 minutes as described above. The percentage of cells staining in the nucleus, cytoplasm, or both compartments was determined.

The results, which are shown in Figure 11B, indicate that mutation of the sequence at 265-267 from KRK to QIL reduced the extent of nuclear localization of NF-ATC in response to ionomycin, but up to 60% of cells show some Ca<sup>2+</sup>-dependent nuclear accumulation of NF-ATC. Mutation of the sequence KRKK (SEQ ID NO: 56) at position 682-685 to TRTG (SEQ ID NO: 55), or precise removal of these 4 residues, had no effect on nuclear localization of NF-ATC in response to Ca<sup>2+</sup> elevation. However, NF-ATc containing mutations in both regions remains cytoplasmic after ionomycin treatment. Thus, like other nuclear proteins with multiple NLSs (Richardson et al. (1986) *Cell*, 44: 77-85), the two NLSs are partially redundant, as suggested by the observation that either can direct cytoplasmic SOS to the nucleus but both must be mutated to prevent nuclear entry. These data also indicated that both NLSs must be inactive in the absence of Ca<sup>2+</sup> stimulations.

**15      Example 11: Mutation of serines in the amino terminus leads to constitutive nuclear localization NF-ATc**

Since the amino terminus of NF-ATc is sufficient for calcineurin-dependent nuclear entry, it was determined whether phosphorylation of the amino terminus directed subcellular compartmentalization of the transcription factor. The amino terminus of each NF-ATc protein contains three copies of a sequence referred to as the SP-repeat motif (Ho et al. (1995) *J. Biol. Chem.*, 270: 19898-19900; Hoey et al. (1995) *Immunity*, 2: 461-472.; Masuda et al. (1995) *Mol. Cell Biol.*, 15:2697-2706). An additional SRR 23 amino acids in length lies just amino-terminal to the first SP repeat (Ho et al. (1995) *J. Biol. Chem.*, 270: 19898-19900) Fig. 9A). Phospho-amino acid analysis revealed that all phosphorylation is located on serines. Two-dimensional tryptic peptide maps show many phosphopeptides derived from the amino-terminal 418 amino acids (see below). To determine whether phosphorylation of these particular serines could regulate NF-ATc localization, selected groups of conserved serines in the SRR and SP repeats were mutated to alanines and the subcellular localization of these mutants was determined in COS cells.

**30**      The SRR was mutagenized by changing the 11 serines to alanines in residues 172-194 to form mSRR. The first SP repeat was mutagenized by changing four serines to alanines in residues 199-211, to form mSP1. The second SP repeat was mutagenized by changing serines at 233 and 237 to alanines, mSP2. The third SP repeat was mutagenized at five serines at 278,

282, 286, 290, and 299, mSP3. Other mutated constructs are shown in Figure 12. These substitutions were made in the construct pSH102cΔ418 (*Nature*, 369:497-502), which encodes the NF-AT carboxy terminal deletion construct (containing amino acid 1- 418) and a hem-Agglutinin (HA) epitope tag at the amino terminus.

5            Immunoblot of cytoplasmic (C) or nuclear (N) extracts of cells transfected with wild-type (WT) or mutant forms of the NF-ATc cDNA were also done. Accordingly, transfected cells were treated for 60 min with media without additions (NS) or with ionomycin and calcium (I + CA<sup>++</sup>), as described above, and then separated into cytoplasmic and nuclear fractions according to *J. Biol. Chem.* 270: 19898-19900, subjected to SDS-PAGE, transferred to a  
10 membrane and a Western blotting was performed using the M2 or 12CA5 antibody that was detected with anti-mouse peroxidase and chemiluminescence (Amersham).

15           The results indicate that mutation of all the serines within the SRR (mSRR) leads to constitutive nuclear localization in 100% of expressing cells that is unaffected by FK506. mSRR has an increased mobility on SDS electrophoresis, is present in the nucleus by Western blotting, and shows reduced incorporation of <sup>32</sup>P after *in vivo* labeling with orthophosphate, consistent with the hypothesis that these serines affect the phosphorylation state of the protein *in vivo*. An NF-ATc mutant in which serines in the first SP repeat were substituted with alanines (mSP1) is also constitutively localized to the nucleus in 100% of expressing cells and shows a reduction in molecular weight. Similar results were obtained in NF-ATc mutants with S→A  
20 mutations in the first and third SP repeat (mSP13) and in versions in which mutations were engineered in all three SP repeats (mSP123) or combined with the mutations in the SRR (mSRR + SP123). The subcellular localization of each of these mutant forms of NF-ATc is constitutively nuclear if they are expressed in Jurkat cells, indicating that these phosphoserines control subcellular localization in a variety of cell types. The unregulated nuclear entry of the  
25 S→A mutations is not likely to be caused by denaturation of the protein, because each of these mutated forms of the mutated forms of NF-ATc participate in NF-ATc-dependent transcription.

30           Since S→A mutation of the SRR resulted in the smallest alteration in apparent molecular weight, it was likely that this region might contain the smallest numbers of critical phosphoserines necessary for cytoplasmic localization. These mutants can be dephosphorylated further after transfection into cells and ionomycin treatment, indicating that the SRR mutant is still a substrate for a phosphatase, possibly calcineurin. Thus further analysis by mutation of smaller blocks of serines in the SRR were performed. Alanine substitution at residues 172-176, 178-181, and 184-188, but not residues 191-194, resulted in nuclear accumulations of NF-ATc

in 100% of expressing cells in the absence of  $\text{Ca}^{2+}$ /calcineurin signaling (see, Figure 12A). Interestingly, the mutants with constitutive nuclear localization remain in the nucleus after adding FK506, a treatment that leads to rapid cytoplasmic accumulation of wild-type NF-ATc, NF-ATp, or NF-ATc3 that has been transported to the nucleus by stimulation (Flanagan et al. 5 (1991) *Nature*, 352: 803-807; Shibasaki et al. (1996) *Nature*, 382: 370-372; Timmerman et al.(1996) *Nature*, 383: 837-840. Thus, these results indicates that phosphorylation of these residues is necessary for export of NF-ATc from the nucleus.

**Example 12: Calcineurin dephosphorylates serines in NF-AT**

10 To determine whether calcineurin could be the phosphatase directing nuclear entry, the ability of calcineurin to specifically dephosphorylate the residues associated with nuclear entry was investigated.

15 NF-AT GST fusion proteins were prepared as follows. Residues 196-304 of NF-ATc1 (Northrop et al. (1994) *Nature*, 369:497-502) were cloned into the *Sma*I site of pGEX-3X to generate pGSP. A GST fusion protein in which the S→A substitutions in all three SP repeats described above was similarly constructed, pGAP, with 9 S and 10 T residues remaining. The 20 GST fusion proteins were phosphorylated by incubating 1  $\mu\text{g}$  of fusion protein immobilized on glutathione-Sepharose with whole brain extract (55  $\mu\text{g}$  protein) (prepared as described below) and with 100  $\mu\text{m}$  ATP and [ $\gamma$ -<sup>32</sup>P]ATP (400  $\mu\text{Ci}/\mu\text{mole}$ ) in 50  $\mu\text{l}$  of kinase buffer (20 mM Tris at pH 7.5, 10 mM  $\text{MgCl}_2$ , 1 mM DTT) for 30 min. at 30°C. Kinase reactions were terminated by 25 washing the agarose beads three times in 1 ml of calcineurin buffer. The fusion proteins were then incubated with calcineurin as above, or treated with 2 units of shrimp alkaline phosphatase (U.S. Biochemical) or 5 units of protein phosphatase 1 (Boehringer Mannheim) in the buffer described by the manufacturer for 30 min at 30°C. Samples were then electrophoresed and exposed for autoradiography.

30 The results, which are presented in Figure 12B, indicate, that once phosphorylated, the 196-304 WT substrate is readily dephosphorylated by *in vitro* treatment with calcineurin and phosphatase I, which is activated by calcineurin (Cohen (1989) *Annual Rev. Biochem.* 58:453). These results indicate that the conserved serines in the SP repeats that control nuclear localization of NF-ATc are substrates for cellular kinases and calcineurin. Glycogen synthase kinase-3 (GSK-3) is a highly conserved proline-directed serine-threonine kinase that phosphorylates NF-AT *in vivo* and opposes  $\text{Ca}^{2+}$ /calcineurin-induced nuclear entry, see below. GSK-3 phosphorylates the conserved serines in the SP repeats *in vitro*. The serines in the SRR

and SP repeat motifs conform to a GSK-3 consensus substrate sequence (Fiol et al. (1994) *J. Biol. Chem.*, 269: 32187-32197). Taken together, these results indicate that the conserved serines in these two motifs are phosphorylated *in vivo* by cellular kinases and dephosphorylated by calcineurin.

5

**Example 13: Phosphoserines in the SRR control an intramolecular interaction within NF-ATc**

The results described above raise the possibility that both basic NLSs interact with phosphoserines on the SP repeats and the serine-rich region to prevent nuclear entry in the unstimulated state. Such intramolecular interactions are difficult to discern because of the difficulty of expressing separate parts of the same protein at concentrations that would be equivalent to the high effective concentration of residues on the same peptide chain. The overcomes this barrier to detecting intramolecular interactions, one part of NF-ATc was immobilized and interactions with other regions expressed in extracts of COS cells, which phosphorylate and translocate NF-ATc under  $\text{Ca}^{2+}$ /calcineurin control were analyzed.

Extracts of COS cells that had been transfected with the empty expression vector or a vector encoding the HA epitope-tagged amino-terminal 418 residues of NF-ATc (2-418) (Northrop (1994) *Nature*, 369:497-502) were incubated with glutathione-agarose beads coupled to GST or incubated with beads coupled to a GST fusion with the RSD of NF-ATc (GST-RSD). A GST fusion protein consisting of the Rel domain of NF-ATc1, GST-RSD (residues 415-716), was expressed in bacteria and affinity purified on glutathione-agarose (*Gene*, 67:31-40). Residues 1-418 of NF-ATc1 tagged at the amino terminus with the HA epitope (*Nature*, 369:497-502) were expressed in COS cells and an extract made by lysis in buffer A (*J. Biol. Chem.* 270: 19898-19900) with protease and phosphatase inhibitors. One hundred micrograms of this extract was incubated with 30  $\mu\text{l}$  of glutathione-agarose coupled to GST, GST-RSD, or GST-mNLS (-2  $\mu\text{g}$  of fusion protein) in 300  $\mu\text{l}$  of incubation buffer (50 mM HEPES at pH 7.8, 150 mM NaCl, 1 mM EDTA, 50 mM NaPO<sub>4</sub>, 0.5% NP-40) with protease and phosphatase inhibitors as in (*J. Biol. Chem.* 270:19898-19900) for 2 hr. at 4°C and washed three times in incubation buffer. Affinity-selected proteins were eluted from the washed beads with SDS sample buffer and detected by immunoblotting using either the 7A6 (*Nature*, 369:497-502) or 12CA5 monoclonal antibodies.

As shown in Figure 13A, when the carboxyl terminus of the protein containing the Rel similarity domain and one of the two partially redundant NLSs was immobilized (GST

415-716), it interacted readily and specifically with the amino-terminal half of NT-ATc1 (1-418) when expressed in COS cell extracts, as well as interacting with the endogenous protein in extracts from lymphocytes.

Because the amino terminus, which contains multiple phosphoserines, might 5 simply interact with basic residues in the Rel similarity region, the NLS in the rel similarity domain was mutated and the binding of this mutated protein to amino-terminal residues 1-418 was analyzed. As shown in Figure 13B, mutation of the carboxy-terminal NLS from KRKK (SEQ ID NO: 56) to TRTG (SEQ ID NO: 55) abolished binding to the amino-terminal 418 residues.

10 This mutation is unlikely to result in denaturation because alteration of this NLS still permits cooperation with NF-ATn and NF-AT-dependent transcription *in vivo*. To demonstrate that this interaction is sensitive to the presence of phosphoserines in the amino terminus, extracts of COS cells that had been transfected with the HA epitope-tagged amino-terminal 418 residues of NF-ATc (1-418 WT) or versions in which S→A mutations were present 15 in the SRR or SP repeats were incubated with GST-RSD and then washed. As shown in Figure 13C, the amino-terminal 418 residues with S→A mutation in the SRR shows reduced association with the carboxyl terminus of the protein, whereas S→A changes in the three SP repeats affect this association less strongly. Each set of mutations results in more rapid migration on SDS electrophoresis, indicating that these S→A mutations prevent phosphorylation. Nonoverlapping 20 S→A mutations within the SRR (Fig. 13C) were also tested in this intramolecular association assay. Alanine substitutions in residues 172-176 reduce the association with the rel similarity domain (RSD), whereas alanine substitutions in serines between 191 and 194 do not alter the association with the RSD. Interestingly, there is a correlation between binding to the RSD in the intramolecular association assay and Ca<sup>2+</sup>/calcineurin-independent nuclear entry--m172-176 is 25 constitutively nuclear and m191-194 undergoes regulated nuclear entry. The differences in the *in vitro* intramolecular association assay between each S→A mutations of NF-ATc are unlikely to be attributable to denaturation, as all mutants are immunoprecipitated by a monoclonal antibody to the region of NF-ATc in the SP repeats (Northrop et al. (1994) *Nature*, 369:497-502), are stable when expressed in cells, and direct NF-AT-dependent transcription. The binding 30 activity is unlikely to indicate a head-to-tail dimer forming between full-length NF-ATc molecules, as the protein is a monomer in solution and when bound to DNA (Hoey et al. (1995) *Immunity*, 2:461-472).

Thus, the interaction within NF-ATc is dependent on residues in the SRR as well

as an intact carboxy-terminal NLS. The correlation between the subcellular localization of mutants in the SRR and their behavior in *in vitro* binding assays suggests that this intramolecular association controls exposure and function of the carboxy-terminal NLS. The S→A changes in the three SP repeats disturbs binding to the RSD only weakly and suggests that these 5 phosphoserines may not participate as strongly in the interaction with the carboxyl terminus of the protein. The dephosphorylation of the SP repeat motifs may result in nuclear localization, by another mechanism, perhaps by exposure of the other NLS that lies between the second and third SP repeats. A model representing the interaction between the NLS and phosphorylated residues in NF-AT is presented in Figure 14. This model is likely to be extended to the other members 10 of the NF-ATc gene family based on the conservation of the NLSs, the SRR, and the SP repeat regions. The amino terminus of NF-ATc3 and NF-ATp (*Proc. Natl. Acad. Sci.*, 93:8907-8912; *Nature*, 382:370-373) undergo  $\text{Ca}^{2+}$ -sensitive nuclear entry.

**Example 14: NF-AT kinase activity copurifies with GSK-3**

15 This example demonstrates that the kinase activity that phosphorylates the N-terminus of NF-AT copurifies with GSK-3.

Protein extracts from rat brains were tested for NF-AT kinase activity as follows. Extracts were prepared from rat brains homogenized in 2 volumes of 20 mM tris (pH 7.5), 1 mM EDTA, 5 mM EGTA, 2 mM dithiothreitol (DTT), and 50 mM  $\beta$ -glycerol-phosphate with 20 protease and phosphatase inhibitors [0.1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM phenylmethylsulfonyl fluoride, pepstatin (1  $\mu\text{g}/\text{ml}$ ), aprotinin (1  $\mu\text{g}/\text{ml}$ ), leupeptin (5  $\mu\text{g}/\text{ml}$ ), and 1 mM benzamidine]. A portion of the 80,000g supernatant was passed over a G-50 sizing column to remove endogenous 25 adenosine triphosphate (ATP), made 10% in glycerol, and used a whole brain extract (5.5mg of protein per ml). The NF-AT kinase activity was followed through  $\text{NH}_4\text{SO}_4$  fractionation and separation on phosphocellulose (P-11 resin), and elution with 200 mM NaCl. The active fractions were pooled and further purified on a Mono-S column (Hughes et al., *Eur. J. Biochem.* 203, 305 (1991)).

30 Column fractions were assayed for NF-AT kinase activity on wild type and mutated NF-AT peptides with [ $\gamma$ -<sup>32</sup>P]ATP and then autoradiographed. Furthermore, since analysis of NF-AT N-terminal portion, in particular amino acids 196-304, indicated the presence of putative overlapping GSK-3 consensus sites (SSXXS(P)) (see Figure 15), column fractions were also assayed for the phosphorylation of GS-2, the GSK-3-specific peptide substrate (Welsh et al., *J. Biol. Chem.* 271, 11410 (1996)). In addition, since GSK-3 (Hughes et al., *Eur. J.*

*Biochem.* 203, 305 (1991)) often phosphorylates serines adjacent to serines previously phosphorylated by protein kinase A (PKA) or another kinase (Fiol *et al.*, *J. Biol. Chem.* 269, 32187 (1994)), phosphorylation of PKA-prephosphorylated wild type NF-AT peptide was also used as a substrate. Phosphorylation of several sites in NF-ATc by PKA could produce a series 5 of phosphorylation-dependent, overlapping GSK-3 consensus sites (Fiol *et al.*, *J. Biol. Chem.* 269, 32187 (1994)) (Fig. 15). The total amount of protein in each fraction was also determined (as measured by absorbance at 280 nm).

The substrate NF-AT peptides were prepared by cloning a DNA fragment encoding residues 196 to 304 of NF-ATc1 (Durand *et al.*, *Mol. Cell. Biol.* 8, 1715 (1988); 10 Cicerill *et al.*, *ibid.* 15, 2071 (1995); Chuvpilo *et al.*, *Nucleic Acids Res.* 21, 5694 (1993); Rooney *et al.*, *Immunity* 2, 473 (1995); Goldfield *et al.*, *J. Exp. Med.* 178, 1365 (1993)) into pGEX-3X to generate pGSP. A GST fusion protein with S→A substitutions (Fig. 15), pGAP, was similarly constructed with 9 serine and 10 threonine residues remaining. Bacterially expressed proteins were purified on glutathione agarose and used at 1  $\mu$ g of fusion protein per 10  $\mu$ l of bead slurry 15 (D. B. Smith and K. S. Johnson, *Gene* 67, 31 (1988)). The fusion proteins were used directly or were prephosphorylated on agarose by addition of 5 units of PKA (Sigma) per microgram of fusion protein at 30°C in kinase buffer [20 mM tris (pH 7.5), 10 mM MgCl<sub>2</sub>, and 1 mM DTT] with 1 mM ATP for 2 hours and then washed to remove PKA and ATP. One unit of PKA is defined as 1 pmol of <sup>32</sup>P transferred per minute. Kinase assays incubated fusion protein (1  $\mu$ g) 20 on glutathione Sepharose, 100  $\mu$ M ATP with [ $\gamma$ -<sup>32</sup>P]ATP (400  $\mu$ Ci/ $\mu$ mol) in 50  $\mu$ l of kinase buffer for 30 min at 30°C. Beads were incubated with 10  $\mu$ l of column fractions or of whole brain extract (55  $\mu$ g of protein), 2.5 units of purified PKA or GSK-3 $\beta$  (New England Biolabs), or both. Experiments with crude or partially purified brain extracts included aprotinin, leupeptin, and pepstatin (all at 1  $\mu$ g/ml), 0.1 mM  $\beta$ -glycerol-phosphate, and 1 mM Na<sub>3</sub>VO<sub>4</sub>. Kinase 25 reactions were terminated by washing the agarose beads twice with 1 ml of TEN [50 mM tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, and 0.5% NP-40] to remove phosphorylated cellular proteins, fractionated on SDS-PAGE, autoradiographed, and stained with Coomassie to ensure that the substrate was not degraded.

The result of the kinase assays using fractions from the column, which are 30 represented in Figure 15 (panels A and B), show that the chromatographic behavior of the NF-AT kinase was similar to that of GSK-3. In particular, NF-AT kinase activity was shown to be strongest in about fractions 35-40 of column P-11 (see Figure 15B) and about fractions 15-25 of Mono-S column (see figure 15C), which are also the fractions which had strongest GSK-3

activity. In fact, the peak of NF-AT kinase activity and GSK-3 immunoreactivity is at fraction 21. Furthermore, the PKA prephosphorylated wild-type NF-AT peptide was also phosphorylated by the same column fractions. On the contrary, the active column fractions did not significantly phosphorylate the mutated NF-AT substrate peptide.

5 Protein immunoblotting with antibodies to GSK-3 $\alpha$  and GSK-3 $\beta$  confirmed that they copurified with the NF-AT kinase (Fig. 15C), and PKA eluted in a partially overlapping peak from the Mono-S column. In fact, the peak of PKA immunoreactivity is at fraction 24. Thus, these results indicate that NF-ATc is likely be a substrate of GSK-3 and PKA.

10 **Example 15: GSK-3 and another kinase synergize to phosphorylate NF-AT**

The role of GSK-3 in the phosphorylation of NF-ATc was assessed by immunodepleting GSK-3 from whole brain extracts. Antisera to GSK-3 $\alpha$  and GSK-3 $\beta$  or control antibodies were used to remove these proteins from whole brain extract. Immunodepletion of GSK-3 activity in 110  $\mu$ g of whole grain extract was done in 200  $\mu$ l of TEN, 1 mM DTT, and 15 protease and phosphatase inhibitors (same as used above) with 3  $\mu$ g of anti-GSK-3 $\alpha$  (sheep polyclonal, Upstate Biotechnolgy), anti-GSK-3 $\beta$  (immunoglobulin G1 (IgG1) monoclonal, Transduction Labs), or both, and 20  $\mu$ l of protein G-Sepharose at 4°C for 4 hours. The IgG1 mouse monoclonal antibody (mAb) M2 (Kodak), sheep polyclonal anti-HIVp17 (NIH), or both 20 were used as control antibodies. The NF-AT kinase assay used 2.5  $\mu$ l of the supernatant (1.2  $\mu$ g of protein) (Cook *et al.*, *EMBO J.* 15, 4526 (1996); Stambolic *et al.* *Curr. Biol.* 6, 1664 (1996)).

25 Immunodepleted extracts were incubated with PKA-prephosphorylated NF-AT in an *in vitro* kinase reaction with [ $\gamma$ -<sup>32</sup>P]ATP, and the <sup>32</sup>P-labeled substrate was detected by autoradiography. Two substrates were used: NF-AT (WT) to detect the priming kinase activity, and WT-PKA prephoshorylated to detect GSK-3 activity. In one reaction, five units of purified GSK-3 $\beta$  were added to the reaction.

30 As shown in Figure 16, depletion of GSK-3 $\alpha$  and GSK-3 $\beta$  from the extracts with specific antibodies completely and specifically removed the NF-AT kinase activity toward NF-ATc prephosphorylated by PKA. However, this immunodepleted extract maintained the ability to phosphorylate NF-ATc (Fig. 16B), which indicated that there are at least two NF-AT kinase activities: an activity that can act directly on NF-ATc, and a second activity that requires prior phosphorylation of NF-ATc. The second kinase activity is that of GSK-3 (as shown by immunodepletion experiments) and the priming kinase activity can be provided *in vitro* by PKA.

However, specific inhibition of PKA in extracts indicates that PKA does not provide all of the priming kinase activity in either brain or lymphocyte extracts. It should be noted, that GSK-3 immunodepletion does not effect the phosphorylation of the unprimed NF-AT substrate because under conditions of substrate excess, only a small percentage of substrate become primed and hence available for subsequent phosphorylation by GSK-3. It is likely that enzyme is limiting in these assays because there is no detectable alteration of the mobility of the substrate upon Coomassie staining, which would reflect phosphorylation.

**Example 16: PKA and GSK-3 stoichiometrically phosphorylate NF-ATc**

The wild-type NF-AT fusion protein (referred to as "WT substrate") was phosphorylated *in vitro* with purified PKA and/or GSK-3 kinases. In the reactions in which the WT substrate was incubated with the two kinases, the first kinase was permitted to phosphorylate the WT substrate with nonradioactive ATP to completion; then, the WT substrate beads were washed to remove the kinase and the WT beads were phosphorylated by the second kinase in the presence of [ $\gamma$ -<sup>32</sup>P]ATP.

The results, which are shown in Figure 17A, indicate that phosphorylation of GSK-3 $\beta$  alone incorporated <0.01 mol of <sup>32</sup>P per mole of NF-AT, whereas PKA alone gave 1 to 2 mol of <sup>32</sup>P per mole of NF-AT and the combination of GSK-3 $\beta$  and PKA gave 3 to 7 mol of <sup>32</sup>P per mole of NF-AT. Casein kinase II (CKII) and Ca<sup>2+</sup>-calmodulin-dependent protein kinase II (CaMkII) did not stoichiometrically phosphorylate the glutathione-S-transferase fusion protein NF-AT-GST. Furthermore, GSK-3 $\beta$  phosphorylated NF-ATc only if it was first phosphorylated by PKA. Similar results were obtained using dephosphorylated NF-ATc purified from lymphocytes as a substrate.

It was then tested whether PKA and GSK-3 $\beta$  contribute to the cellular phosphorylation of NF-ATc by comparing the tryptic phosphopeptides from NF-ATc phosphorylated *in vivo* with those derived from *in vitro* phosphorylation of the NF-AT fusion protein. NF-ATc was overexpressed in COS cells (which support reversible Ca<sup>2+</sup>-dependent nuclear localization) and labeled with [<sup>32</sup>P]orthophosphate. COS cells transfected with 3  $\mu$ g of PSH102 (Northrop *et al.*, *Nature* 369, 497 (1994)) were labeled with [<sup>32</sup>P]orthophosphate (1 mCi/ml) for 6 hours and immunoprecipitated with the hemagglutinin (HA) mAb 12CA5, transferred to polyvinylidene difluoride membrane, and digested with trypsin. Oxidized peptides (1000 cpm) were separated by electrophoresis on cellulose at pH 1.9 for 30 min at 1000 V and then chromatographed in the second dimension using butanol-acetic acid-pyridine solvent (Boyle

et al., *Methods Enzymol.* 201, 110 (1991)). In one reaction, the PKA + GSK-3 $\beta$  *in vitro* phosphorylated peptides were mixed with the *in vivo* phosphorylated peptides before two-dimensional separation to establish that they are similar.

5 The results, shown in Figure 17B, indicate that the tryptic phosphopeptides from NF-ATc phosphorylated *in vivo* with those derived from *in vitro* phosphorylation of the NF-AT fusion protein were identical, with the exception of one phosphopeptide. These results suggest that GSK-3 $\beta$  and another kinase synergize to phosphorylate NF-AT on the sites involved in Ca<sup>2+</sup>-dependent nuclear localization *in vivo*.

10 **Example 17: Sites of phosphorylation of NF-T by PKA and GSK-3**

The sites of phosphorylation by GSK-3 and PKA were defined by Edman degradation of *in vitro* <sup>32</sup>P-labeled tryptic fragments. Wild-type NF-ATc-GST fusion protein was phosphorylated *in vitro* with PKA with [ $\gamma$ -<sup>32</sup>P]ATP(50 $\mu$ Ci/ $\mu$ mol) and cleaved with Factor Xa to release the fusion protein. This was isolated on SDS-polyacrylamide gel electrophoresis (PAGE) and cleaved by trypsin, and radioactive fragments were purified by high-performance liquid chromatography (HPLC). One radioactive fraction released <sup>32</sup>P in the second Edman degradation cycle and had the sequence ASVTEESWLGAR (SEQ ID NO: 83) of the tryptic peptide with Ser<sup>245</sup> in the second position. A second radioactive fraction released <sup>32</sup>P in the third Edman degradation cycle and had a molecular size indicating the tryptic peptide KYSLNGR encompassing Ser<sup>269</sup> in the NF-ATc sequence. A second GST fusion protein encoding residues 223 to 277 of NF-AT was purified, phosphorylated *in vitro* with nonradioactive ATP and PKA, washed, then phosphorylated with [ $\gamma$ -<sup>32</sup>P]ATP (50 $\mu$ Ci/ $\mu$ mol) and GSK-3 $\beta$ . The fusion protein was isolated on SDS-PAGE and cleaved by trypsin, and two radioactive fragments were purified by HPLC. One radioactive fragment contained the tryptic peptide GLGACTLLGSPQHSPSTSPR (SEQ ID NO: 84).

20 Thus, the results indicate that PKA phosphorylates the NF-ATc fusion protein at two serines (Fig. 15A). The PKA site at Ser<sup>245</sup> creates a series of overlapping GSK-3 substrate sites. Phosphorylation of the PKA-prephosphorylated NF-ATc fusion protein by GSK-3 $\beta$  labeled the peptide that contains this array of GSK-3 sites (Fig. 15A).

25

**Example 18: GSK-3 overexpression blocked Ca<sup>2+</sup> NF-AT induced nuclear translocation**

The biological importance of NF-ATc phosphorylation by GSK-3 $\beta$  was assessed

by manipulating its activity in cells and determining the effect on the subcellular localization of NF-ATc. Cos cells, which like many cells, express GSK-3 (Woodgett, in *Methods in Enzymology*, T. Hunter and B. M. Sefton, Eds. (Academic Press, San Diego, CA, 1991), vol. 200, p. 564), were cotransfected with 1  $\mu$ g of a construct encoding FLAG epitope-tagged NF-ATc1 5 and 3  $\mu$ g of GSK-3 expression vector or with the empty vector and the cells were left unstimulated or were treated with 2  $\mu$ M ionomycin and 10 mM CaCl<sub>2</sub> (I + Ca<sup>2+</sup>) to induce nuclear localization of NF-ATc. Human GSK-3 $\beta$  cDNA (He et al., *Nature* 374, 617 (1995)) was cloned into pBJ-5. NF-ATc was visualized with FLAG mAb M2 and indirect immunofluorescence. COS cell NF-AT translocation assay were done as described in Northrop 10 et al., *Nature* 369, 497 (1994).

The results show that transfected NF-ATc family members (Shibasaki et al., *Nature* 382, 370 (1996); Luo et al., *Proc. Natl. Acad. Sci. U.S.A.* 93, 8907 (1996)), like endogenous NF-ATc, 15 were cytoplasmic and translocated to the nucleus when cells were stimulated by agents that increase intracellular Ca<sup>2+</sup>. Furthermore, overexpression of GSK-3 $\beta$  blocked the Ca<sup>2+</sup>- calcineurin-induced nuclear translocation of coexpressed NF-ATc in COS cells.

In another example, endogenous NF-AT-dependent transcription was shown to be inhibited by overexpression of GSK-3 $\beta$ . Jurkat-T antigen cells were transfected with 2  $\mu$ g of a transcription reporter plasmid (NF-AT dependent reporter, AP-1 dependent reporter, or HIV-LTR containing reporter linked to a gene encoding the SEAP) and either 3  $\mu$ g of the GSK-3 $\beta$  expression construct or empty vector. NF-AT SEAP activity was measured and expressed as a 20 percentage of the ionomycin-stimulated and phorbol 12-myristate 13-acetate (PMA)-stimulated control activity; AP-1 and HIV-LTR SEAP activities are expressed as a percentage of PMA-stimulated activity (Spencer et al., *Science* 262, 1019 (1993)). The results, which are depicted 25 in Figure 18, panel A, show that GSK-3 overexpression inhibits NF-AT dependent reporter gene expression. AP-1 dependent reporter gene expression was also downregulated by GSK-3 overexpression, probably due to the ability of GSK-3 to produce an inhibitory phosphorylation on c-Jun (Mikolakaki et al., *Oncogene* 8, 833 (1993)). GSK-3 did not have an inhibitory effect on the HIV-LTR dependent reporter gene expression.

In yet another example, the ability of various serine-threonine kinases to inhibit the 30 nuclear entry of cotransfected NF-ATc in COS cells was compared. Accordingly, COS cells were cotransfected with 1  $\mu$ g of FLAG epitope-tagged NF-ATc1 and 1  $\mu$ g of the serine-threonine kinases CKII, CaMk $\delta$ A, CaMk $\delta$ B, PKA or PKC or 3  $\mu$ g of GSK-3 $\beta$  or 0.5  $\mu$ g of ERK. ERK cDNA was cloned into pBJ-5. *Drosophila* CKII cDNA was polymerase chain reaction-

amplified and cloned into pBJ-5. Murine PKA cDNA and an activated form of PKC- $\beta$  were cloned into pSR $\alpha$ . The calcineurin A and B expression constructs (Clipstone and Crabtree, *Nature* 357, 695 (1992)), CaMkII constructs (Srinivasan et al. *J. Cell Biol.* 126, 839 (1994)), COS cell NF-AT translocation assay, and Jurkat -T antigen cell transcription reporter assays (Northrop et al. (1994) *Nature* 369:497) were as described. Transfected cells were stimulated with ionomycin and 10 mM Ca<sup>2+</sup>, and the percentages of cells expressing NF-AT localized in the nucleus, cytoplasm, or both compartments were scored visually and are presented as a percentage of expressing cells. The transfected ERK kinase was activated by adding PMA (25 ng/ml). Comparison of the relative expression of the HA epitope-tagged kinases was performed by 10 immunoblotting 15  $\mu$ g of whole cell extracts with HA mAb 12CA5.

The results, which are presented in Figure 18 B, indicate that although GSK-3 was expressed in smaller amounts, it was the most active in inhibiting nuclear entry of NF-ATc. Overexpression of PKA had little effect on NF-ATc localization. This may indicate that endogenous PKA activity or another kinase is adequate to phosphorylate NF-ATc in COS cells 15 or that such phosphorylation is necessary, but not sufficient, for nuclear export. Thus, these results indicate that the Ca<sup>2+</sup>-calcineurin signaling pathway is opposed by GSK-3.

#### **Example 19: Overexpression of GSK-3 enhances the nuclear export of NF-AT**

This example describes the effects of GSK-3 on the nuclear export of NF-AT by 20 first causing its translocation to the nucleus by stimulating cells with ionomycin, then removing the Ca<sup>2+</sup>-calcineurin signal and blocking further nuclear import with the calcineurin inhibitor FK506 (Clipstone and G. R. Crabtree, *Nature* 357, 695 (1992)).

COS cells were cotransfected with expression constructs encoding FLAG epitope-tagged NF-ATc1 (1  $\mu$ g), calcineurin A and B (0.5  $\mu$ g each) (N. A. Clipstone and G. R. Crabtree, *Nature* 357, 695 (1992)), and 2  $\mu$ g of vector, GSK-3 $\beta$  or GSK-KM, a catalytically inactive GSK-3 $\beta$  (He et al., *Nature* 374, 617 (1995)). Cells were also cotransfected with a version of NF-ATc1 in which the underlined serines in Fig. 15A were changed to alanines with calcineurin and GSK-3 $\beta$ . The inclusion of Ca-calcineurin promotes NF-ATc nuclear entry (Shibasaki et al., *Nature* 382, 370 (1996); Luo et al., *Proc. Natl. Acad. Sci. U.S.A.* 93, 8907 (1996)) and 25 overcomes the cytoplasmic localization of NF-ATc induced by GSK-3 $\beta$  overexpression. Wild-type NF-ATc was localized in the cytosol in 98% of unstimulated expressing cells, whereas 90% of cells translocated NF-ATc to the nucleus with I + Ca<sup>2+</sup> treatment; this translocation was 30 completely blocked by FK506. NF-ATc was localized in the nucleus by treatment with I + Ca<sup>2+</sup>

för 60 min, then the medium was changed to medium with FK506 (20 ng/ml) to terminate  $\text{Ca}^{2+}$  signaling and to block nuclear reentry of NF-ATc. Transfected NF-ATc was detected with FLAG mAb M2 by indirect immunofluorescence, and 200 expressing cells were scored as expressing NF-ATc in the cytoplasm, nucleus, or both compartments.

5 The results, shown in Figure 19, show that overexpression of GSK-3 $\beta$  in amounts approximately one-tenth those of NF-ATc enhanced the movement of NF-ATc into the cytoplasm relative to that in cells transfected with the vector or with a catalytically inactive form of GSK-3 $\beta$  (He et al., *Nature*, 374, 617 (1995)). GSK-3 $\beta$  overexpression did not influence the constitutive nuclear localization of NF-ATc with S-A mutations in the serine-proline repeats.

10 These data indicate that GSK-3 $\beta$  acts catalytically to direct the nuclear export of NF-ATc and that the regulation of nuclear export involves the phosphorylation of NF-ATc at conserved serines. Because NF-ATc family members are expressed in many tissues and have sequence similarity at the  $\text{NH}_2$ -terminal residues involved in nuclear import and export, GSK-3 is likely to control the compartmentalization of each of the four different NF-ATc family members.

15

**Example 20: NF-AT is a therapeutic target for cardiac hypertrophy**

**The  $\text{Ca}^{++}$  /Calcineurin/NF-AT signaling pathway.**

The NF-AT transcription factor was initially described in T cells as a rapidly inducible protein complex binding to the distal antigen receptor response element, ARRE-2, of the human IL2 promotor (1,2). The active transcription factor is made up of cytosolic (NF-ATc) and nuclear components (NF-ATn) (3). The NF-ATc family of transcription factors is encoded by at least four distinct genes, NF-ATc1, c2, c3, c4 (Genome Data Base (GBD) Nomenclature Committee) (4-9). NF-ATc family proteins show specific patterns of tissue distribution (Table 25 1).

Table 1

| <i>GBD Name</i> | <i>Tissue Distribution</i>   | <i>K.O. Phenotype</i>                                  |
|-----------------|--|--|
| NF-ATc1         | Lymphocytes, Heart valves, Muscle, Prostate, Colon, Parathyroid    | Failure of heart valve development<br>IL4, IL2 reduced |
| 30 NF-ATc2      | Lymphocytes, Brain, Pancreas, Testis, Placenta                     | Proliferation of lymphocytes enhanced<br>IL4 enhanced  |
| NF-ATc3         | Lymphocytes, Muscle, Heart, Kidney, Brain, Skin                    | Proliferation of lymphocytes enhanced<br>IL4 enhanced  |
| NF-ATc4         | Heart, Lung, Lymphocytes, Kidney, Brain (hippocampus, cerebellum), | N.A.   |

Activation of NF-AT-dependent transcription integrates at least three different signaling pathways. NF-ATc is sequestered in the cytoplasm and rapidly translocates to the nucleus in response to a rise in  $[Ca^{++}]_i$  (3,10-12). This cytoplasmic to nuclear translocation requires dephosphorylation of NF-ATc by calcineurin and is blocked by the immunosuppressive drugs cyclosporin A (CsA) and FK506, which selectively inhibit the activity of calcineurin (13-21).  
5 Induction and/or activation of NF-ATn requires signals from the Ras/MAPK pathway (7,10,22) and Rac/CDC42-dependent reorganization of the cytoskeleton ((23)). Each NF-ATc family member contains two main functional domains, an N-terminal regulatory region or NF-AT Homology Region (NHR) and a C-terminal DNA Binding Domain (DBD) or Rel Homology Domain (RHD). The N-terminal regulatory domain is necessary and sufficient for 10 regulated nuclear import and export of NF-ATc proteins. Within the N-terminal region there are several conserved motifs, including a serine rich region (SRR) and three serine-proline (SP) repeats, which are proline-directed kinase sites (8,19,24). These motifs are the target of bidirectional NF-ATc regulation by opposing kinases and phosphatases. The conserved phospho-serines in these motifs serve as substrates for calcineurin, while the serine-threonine kinase glycogen synthase kinase-3 (GSK-3) phosphorylates NF-ATc at these conserved serine residues and opposes the  $Ca^{++}$ /calcineurin induced nuclear entry (24). The highly conserved C-terminal DNA binding domain, which shows moderate sequence homology to and shares the topology with the DNA-binding domain of Rel-family proteins, does not bind to DNA by itself 15 at physiologic concentrations due to substitutions at critical residues (25-30). Thus, all NF-ATc proteins require a nuclear partner (NF-ATn) for binding to DNA (10). Heterodimeric combinations of the AP-1 family members, Fos and Jun, have been shown to function as NF-ATn (25,30,31). NF-ATc and AP-1 cooperatively bind to juxtaposed recognition sites and synergistically activate gene expression (25,28,32). Additionally the zinc finger transcription 20 factor GATA4 has been shown to cooperate with NF-ATc4 in the activation of transcription in cardiomyocytes (36).  
25

### Cardiac Hypertrophy

In the United States half a million new cases of heart failure are diagnosed each year, 30 with a mortality rate of about 50%. Cardiac hypertrophy is a generalized enlargement of the myocardium and is initially a compensatory response of the heart muscle that augments cardiac output. However, sustained hypertrophy can lead to dilated cardiomyopathy, functional

insufficiency, heart failure and sudden death. A variety of underlying disease states such as hypertension, myocardial infarction, cardiac arrhythmias, endocrine disorders and genetic mutations of cardiac proteins (33-35) genes can lead to hypertrophic cardiomyopathy (HCM). Overexpression of a constitutively active /Ca<sup>++</sup> independent form of calcineurin or a 5 constitutively nuclear form of NF-ATc4 in the heart of transgenic mice evoke molecular and pathophysiologic changes typical of cardiac hypertrophy and mimic all pathological aspects of human heart failure (36). The development of HCM in the calcineurin transgenic animals is blocked by treatment with Cyclosporin A (CsA), demonstrating that the phosphatase activity of calcineurin is essential for the induction of HCM. Stimulation of cardiomyocytes with 10 angiotensin II and phenylephrine, which induce hypertrophic responses in cardiomyocytes, activate NF-AT-dependent transcription in cardiomyocytes. Furthermore, CsA and FK506 block the angiotensin and phenylephrine induced hypertrophic response *in vitro*. CsA has also been shown to block the development of HCM in three mouse models based on mutations in contractile heart proteins such as tropomodulin, myosin light chain-2, fetal  $\beta$ -tropomyosin (33). 15 Additionally, CsA and FK506 treatment prevented pressure overload induced hypertrophy caused by aortic banding of rats(33). These data indicate that the Ca<sup>++</sup> /calcineurin/NF-ATc signaling pathway may play a crucial role in the initiation of HCM. All hypertrophic stimuli increase intracellular Ca<sup>++</sup> levels (37-39). This increase in intracellular Ca<sup>++</sup> could directly activate calcineurin. Activation of calcineurin could initiate HCM by dephosphorylation and 20 nuclear translocation of NF-ATc proteins. NF-ATc dependent transcription could induce hypertrophic response genes.

Voltage gated Ca<sup>++</sup> channels (VSCCs) play a central role in the development and the contractility of the heart muscle. VSCCs open by membrane depolarization during the fast upstroke of the action potential. Prolongation of action potential duration with delayed 25 repolarization and alterations in Ca<sup>++</sup> homeostasis are common findings in human heart failure and animal models of cardiac hypertrophy. Cardiac myocytes express the high voltage-activated L-type VSCCs and the low voltage-activated T-type VSCCs. L-type Ca<sup>++</sup> channels play a central role in the excitation-contraction coupling in cardiac muscle and thus are key regulators of inotropy. Ca<sup>++</sup> influx via L-type VSCCs induces Ca<sup>++</sup> release from the 30 sarcoplasmic reticulum (SR) during the systole and initiates contraction. In cardiac muscle, Ca<sup>++</sup> influx through VSCCs is closely associated with the action of catecholamines.  $\beta$ -adrenergic stimulation of cardiomyocytes increases the opening probability of L-type VSCCs

vía increased levels of intracellular cAMP. Additionally,  $\beta$ -adrenergic stimulation of the cAMP-dependent protein kinase A pathway increases the affinity of the SR  $\text{Ca}^{++}$ -ATPase for  $\text{Ca}^{++}$  via phosphorylation of the regulatory protein phospholamban, thus increasing the  $\text{Ca}^{++}$  filling status of the SR. The filling status of the SR determines the force of contraction during 5 the next systole. Additionally,  $\text{Ca}^{++}$  influx via L-type VSCCs can trigger gene transcription in neurons (40) and skeletal muscle (41). T-type-channels in the heart are present at lower density than L-type channels. The role of T-type channels is not as well defined. T-type channels are thought to play a role in the rhythmic activity of the heart, during embryonic heart development and potentially during pathological conditions such as hypertrophy (42).

10

### Research Results

We have used homologous recombination to delete exon 1, 2 and part of exon 3 of the NF-ATc4 gene, coding for amino acid 1-438 of the NF-ATc4 protein which has been 15 implicated by Dr Eric Olson's group in cardiac hypertrophy. A 15 kilobase genomic clone obtained from a mouse 129 library was used to construct a targeting vector for homologous recombination by positive/negative selection. A PGK-neo cassette was inserted in inverse orientation into the first, second and third exon of the NF-ATc4 gene, deleting the regulatory region and part of the DNA binding domain of NF-ATc4 (Figure 1). Targeted ES cell clones 20 were used for microinjection into C57Bl/6 blastocysts, followed by uterine transfer into pseudopregnant CD-1 females. Male chimeric mice were mated to CD-1, C57Bl/6 and 129Sv female mice and the resulting heterozygous mice were bred to homozygosity. The homozygous NF-ATc4 knock-out mice are viable and hence can be tested for cardiac function.

25

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30

### Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

**CLAIMS**

1. A method for preventing or reducing cardiac hypertrophy in a subject, comprising administering to the subject a pharmaceutically effective amount of an NF-AT antagonist to decrease the biological activity of NF-AT in myocardial tissue, to thereby prevent or reduce cardiac hypertrophy in the subject.
- 5 2. The method of claim 1, wherein the NF-AT antagonist decreases the transcriptional activity of NF-AT.
- 10 3. The method of claim 2, wherein the NF-AT antagonist inhibits the nuclear translocation of NF-AT.
4. The method of claim 3, wherein the NF-AT antagonist inhibits dephosphorylation of NF-AT.
- 15 5. The method of claim 4, wherein the NF-AT antagonist inhibits binding of calcineurin to NF-AT.
6. The method of claim 3, wherein the NF-AT antagonist stimulates phosphorylation of NF-AT.
- 20 7. The method of claim 6, wherein phosphorylation of NF-AT is stimulated by increasing GSK-3.
8. The method of claim 2, wherein the NF-AT antagonist inhibits the formation of a complex comprising NF-AT.
9. The method of claim 7, wherein the complex comprises a nucleic acid comprising an NF-AT binding site.
10. The method of claim 9, wherein the NF-AT antagonist is a dominant negative mutant of an NF-AT polypeptide.
- 25 11. The method of claim 9, wherein the NF-AT antagonist is a nucleic acid encoding a dominant negative mutant of an NF-AT polypeptide.
12. The method of claim 1, wherein the NF-AT antagonist inhibits the production of an NF-AT polypeptide.
13. The method of claim 12, wherein the NF-AT antagonist is an antisense NF-AT nucleic acid or a ribozyme.
- 30 14. The method of claim 12, wherein the NF-AT antagonist inhibits transcription of a gene encoding an NF-AT polypeptide.
15. The method of claim 1, wherein the NF-AT antagonist is delivered locally.
16. The method of claim 1, wherein the NF-AT antagonist is in a pharmaceutically acceptable delivery vehicle which is targeted to the heart.
- 35 17. The method of claim 1, wherein the subject has congestive heart disease.
18. The method of claim 1, wherein NF-AT is NF-ATc4.
19. The method of claim 18, wherein the NF-AT antagonist is an antagonist of an NF-ATc4 polypeptide, and the antagonist is not an antagonist of NF-ATc1, NF-ATc2 and NF-ATc3 polypeptide.
- 40 20. The method of claim 1, wherein the antagonist is identified by a method comprising

- (i) contacting an isolated NF-AT polypeptide or portion thereof sufficient for interacting with a molecule, with the molecule and a compound in conditions under which, but for the presence of the compound, the NF-AT polypeptide or portion thereof and the molecule interact; and
- 5 (ii) determining the level of interaction between the NF-AT polypeptide or portion thereof and the molecule in the presence relative to the absence of the compound, such that a weaker interaction between the NF-AT polypeptide or portion thereof and the molecule in the presence, relative to the absence, of the compound indicates that the compound is an antagonist of the activity of an NF-AT polypeptide.
- 10 21. The method of claim 20, wherein the molecule is a nucleic acid.
- 22. The method of claim 21, wherein the nucleic acid comprises an NF-AT recognition sequence.
- 23. The method of claim 20, wherein the molecule is a polypeptide.
- 24. The method of claim 23, wherein the polypeptide is a leucine zipper containing polypeptide.
- 15 25. The method of claim 24, wherein the polypeptide is c-Fos or c-Jun.
- 26. The method of claim 23, wherein the polypeptide is calcineurin.
- 27. The method of claim 20, wherein the NF-AT polypeptide is NF-ATc4 and the method further comprises selecting against compounds which inhibit NF-AT polypeptides other than NF-ATc4.
- 20 28. The method of claim 1, wherein the antagonist is identified by a method comprising
  - (i) contacting a cell comprising an NF-AT polypeptide and a reporter gene operably linked to a promoter comprising an NF-AT binding site with a test compound; and
  - 25 (ii) determining the level of expression of the reporter gene in cells that were contacted with the test compound relative to cells that were not contacted with the test compound, such that a lower level of expression of the reporter gene in cells contacted with the test compound relative to cells that were not contacted with the test compound indicates that the compound is an antagonist of NF-AT activity.
- 30 29. The method of claim 1, wherein the antagonist is identified by a method comprising
  - (i) contacting an NF-AT polypeptide or portion thereof that can be phosphorylated with a mixture and a compound in conditions under which, but for the compound, phosphorylation of NF-AT is modulated by the cell extract; and
  - 35 (ii) determining the level of phosphorylation of the NF-AT polypeptide or portion thereof in the presence relative to the absence of the compound, such that a higher level of phosphorylation of the NF-AT polypeptide or portion thereof in the presence relative to the absence of the compound indicates that the compound is an antagonist of NF-AT activity.
- 40 30. The method of claim 1, wherein the antagonist is identified by a method comprising
  - (i) contacting a cell comprising an NF-AT polypeptide or portion thereof that can be

phosphorylated with an agent which modulates phosphorylation of NF-AT and a compound; and

5 (ii) determining the level of phosphorylation of the NF-AT polypeptide or portion thereof in cells contacted with the compound, relative to cells that were not contacted with the compound,

such that a lower level of phosphorylation of the NF-AT polypeptide or portion thereof indicates that the compound is an antagonist of NF-AT activity.

31. The method of claim 1, wherein the antagonist is identified by a method comprising

10 (i) contacting a cell comprising an NF-AT polypeptide, or portion thereof sufficient for translocation from the cytoplasm to the nucleus, with a compound which stimulates translocation of an NF-AT polypeptide from the cytoplasm to the nucleus and with a test compound; and

(ii) determining the cellular location of the NF-AT polypeptide after step (i),

15 such that the presence of increased levels of the NF-AT polypeptide in the cytoplasm of the cell contacted with the test compound relative to a cell that was not contacted with the test compound indicates that the test compound is an antagonist of NF-AT activity.

32. The method of any one of claims 28-31, wherein the NF-AT polypeptide is NF-ATc4 and the method further comprises selecting against compounds which inhibit NF-AT polypeptides other than NF-ATc4.

20

33. A pharmaceutical composition for treating or preventing cardiac hypertrophy comprising an NF-AT antagonist in a pharmaceutically acceptable delivery vehicle for targeting the NF-AT antagonist to the heart.



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| 1410  | 1430 | 1450 | 1470 | 1490 |
|---|------|------|------|------|
| 388 A V P Q H P Y Q W A K P K Y L S P T S T M S P T I P A L D W Q L P S   | 421  |      |      |      |
| 1510  | 1530 | 1550 | 1570 | 1590 |
| 422 H S C P Y E L K I E V Q P K S H I I K A H Y E T E G S R G A Y K A S   | 454  |      |      |      |
| 1610  | 1630 | 1650 | 1670 | 1690 |
| 455 A G G H P I V Q I H G Y L E N E P I H L O L F I C T A D D R L L R     | 487  |      |      |      |
| 1710  | 1730 | 1750 | 1770 | 1790 |
| 488 P H A F Y U V H K I T G K T V S T T S H E A I L S N T K V L E I P L   | 521  |      |      |      |
| 1810  | 1830 | 1850 | 1870 | 1890 |
| 522 L P E N S H R A V I D C A C I L K I R N S D I F I R K G E T D I G     | 554  |      |      |      |
| 1910  | 1930 | 1950 | 1970 | 1990 |
| 555 B K N T R V R L V F R Y H V P Q P S G R T I S L O V A S N P I E C     | 587  |      |      |      |
| 2010  | 2030 | 2050 | 2070 | 2090 |
| 588 S Q R S A Q E J Y L V E K Q S T D S T P Y V V G G K K M V L S G H N F | 621  |      |      |      |
| 2110  | 2130 | 2150 | 2170 | 2190 |
| 622 L Q D S K V T F V E K A P D G H H V W E M E A K T D R D L C K P N     | 654  |      |      |      |
| 2210  | 2230 | 2250 | 2270 | 2290 |
| 655 S L V V E I P H F R N Q R I T S P V H V S F T V C N G K K K R S Q     | 687  |      |      |      |
| 2310  | 2330 | 2350 | 2370 | 2390 |
| 688 Y O R F T Y L P A N G N A I F L T V S R E H E R V G C F Y             | 716  |      |      |      |
| 2410  | 2430 | 2450 | 2470 | 2490 |
| 2510  | 2530 | 2550 | 2570 | 2590 |
| 2610  | 2630 | 2650 | 2670 | 2690 |
| 2710  | 2730 | 2750 |      |      |

FIG. 1B

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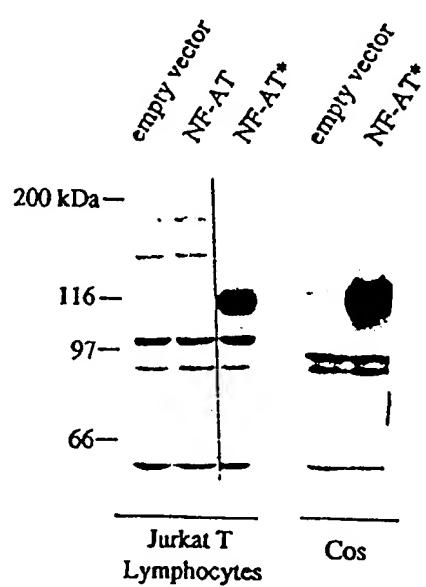


FIG. 2.

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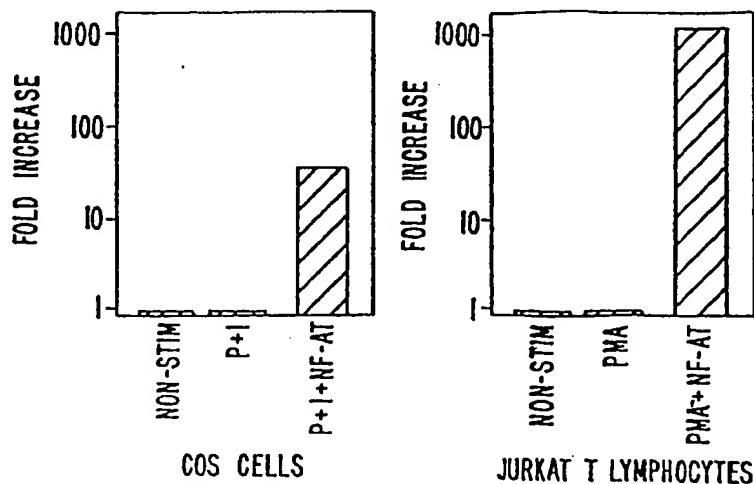


FIG. 3A.

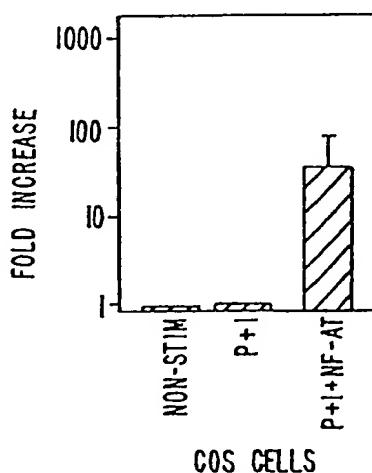


FIG. 3B.

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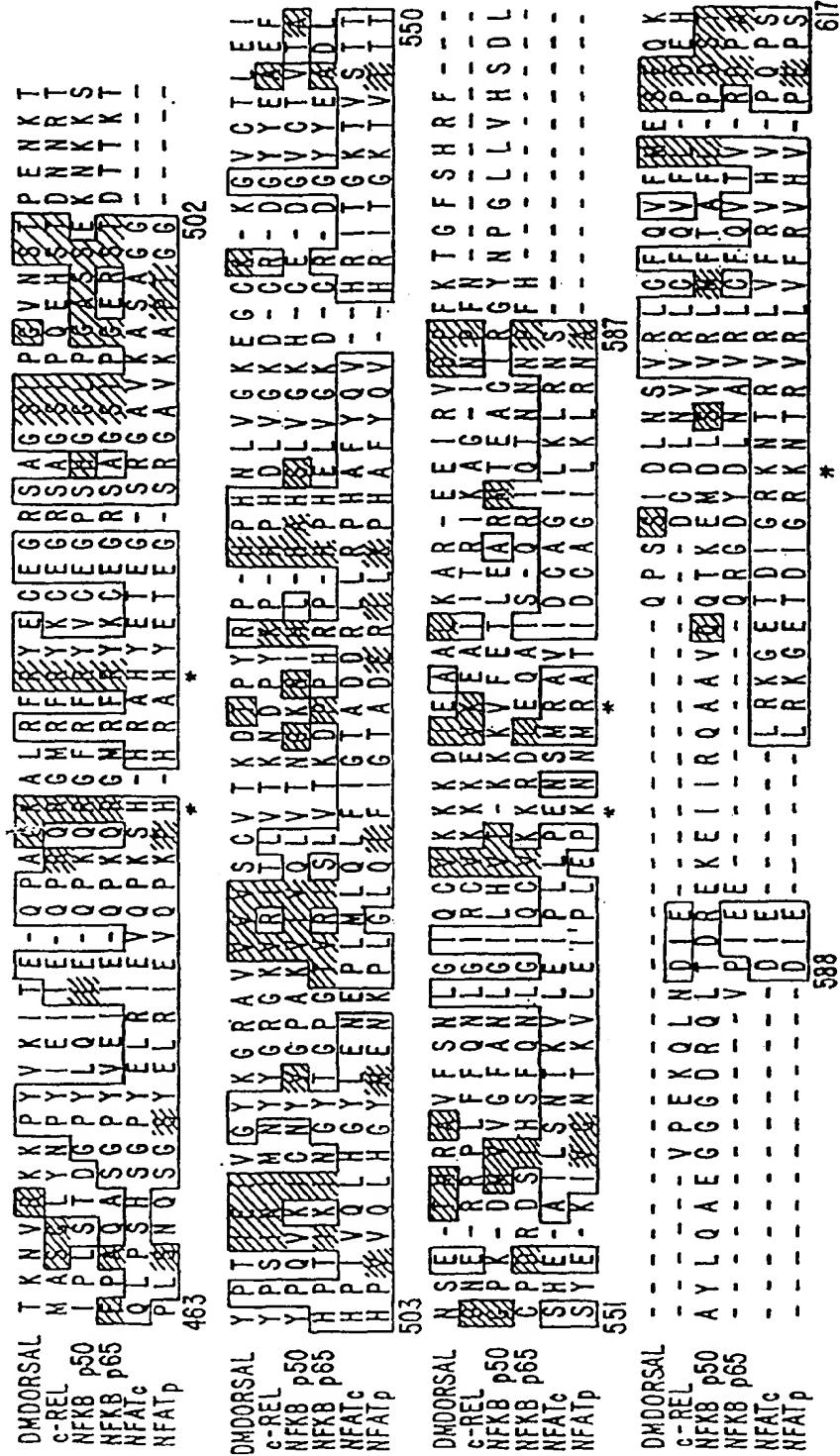


FIG. 4-A

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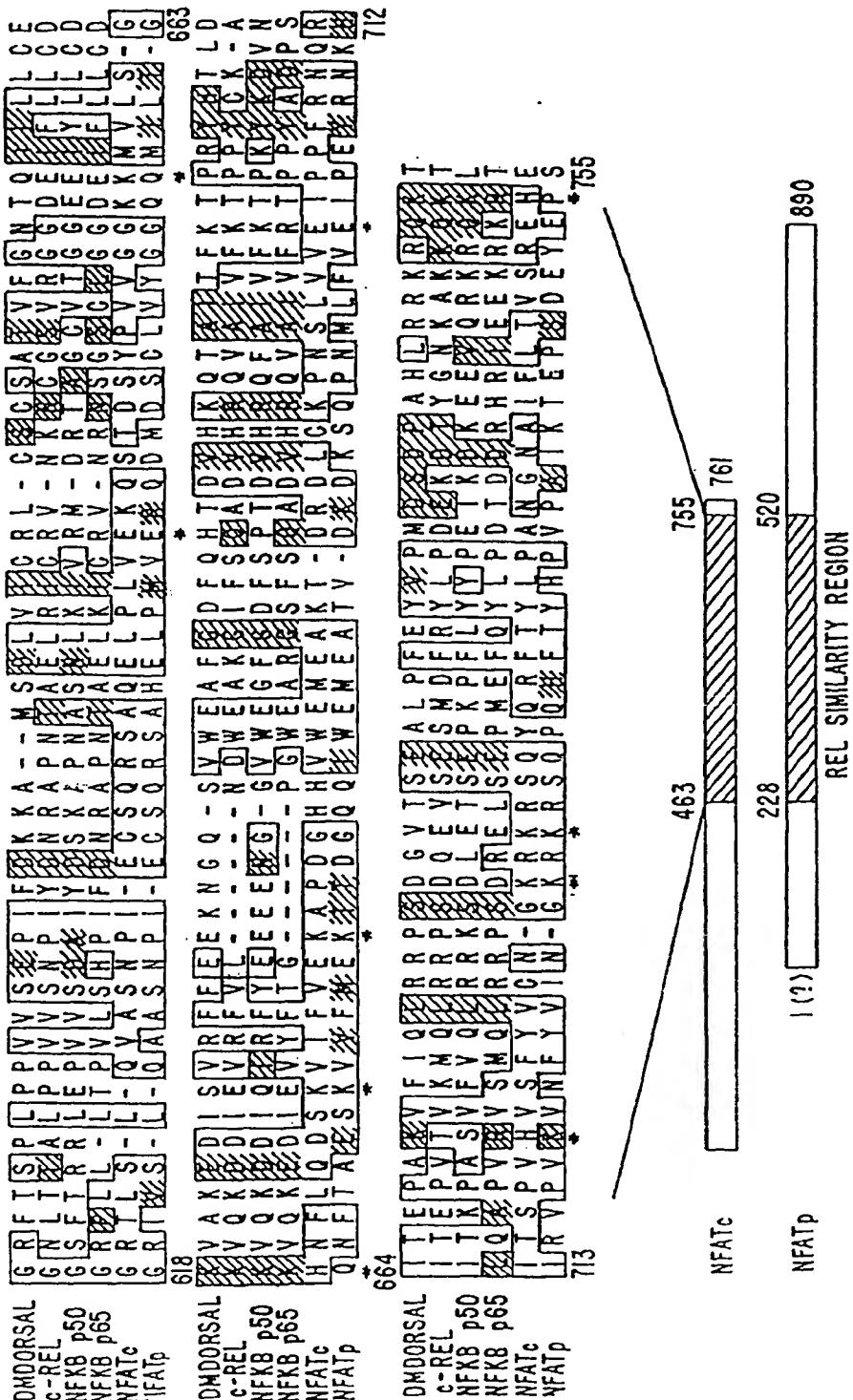


FIG. 4-B

REL SIMILARITY REGION

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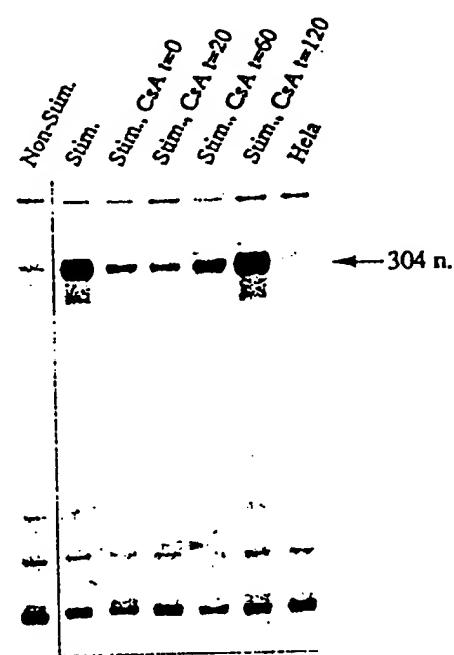


FIG. 5A.

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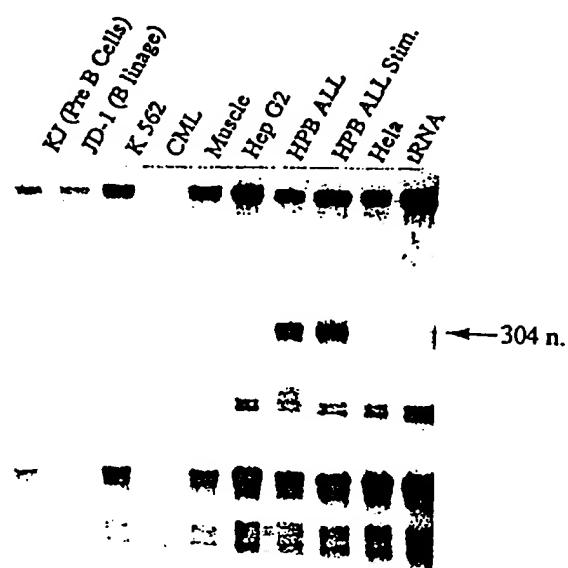


FIG. 5B.

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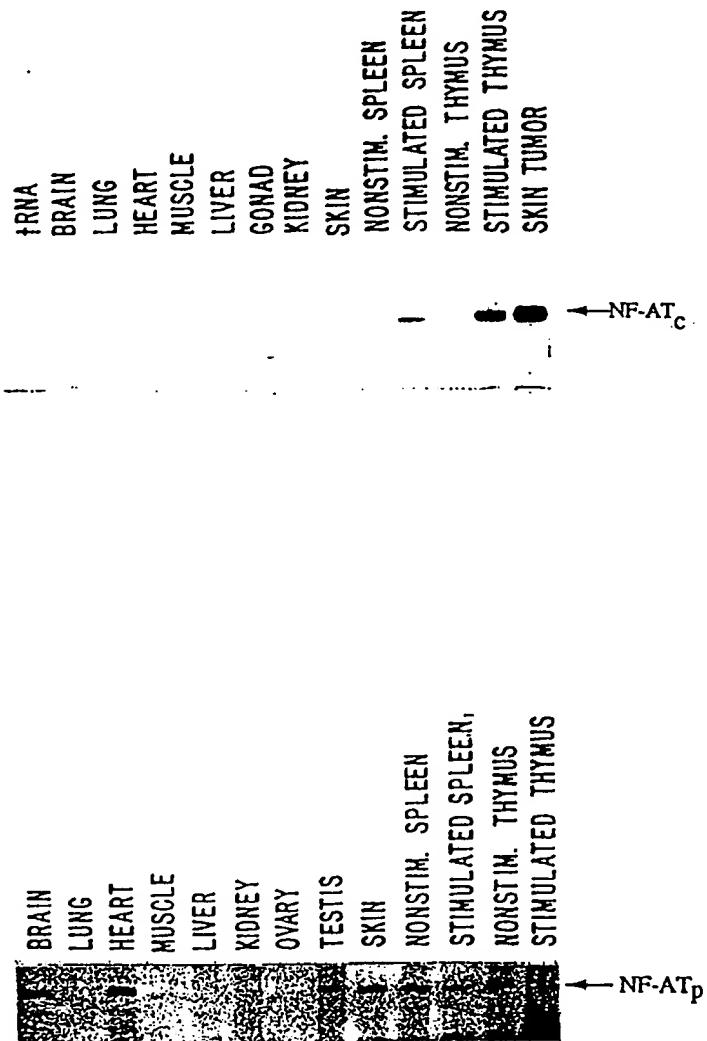


FIG. 5C.

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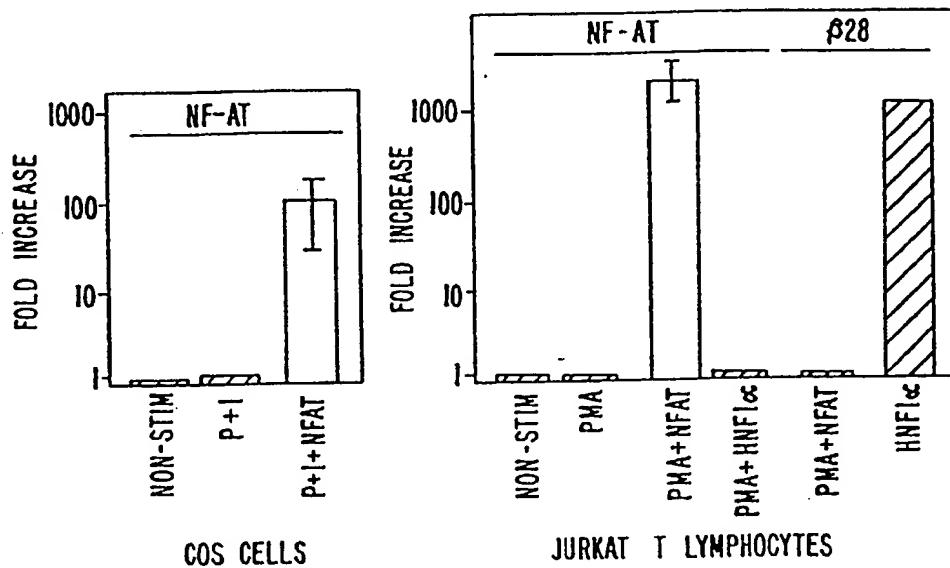


FIG. 6A.

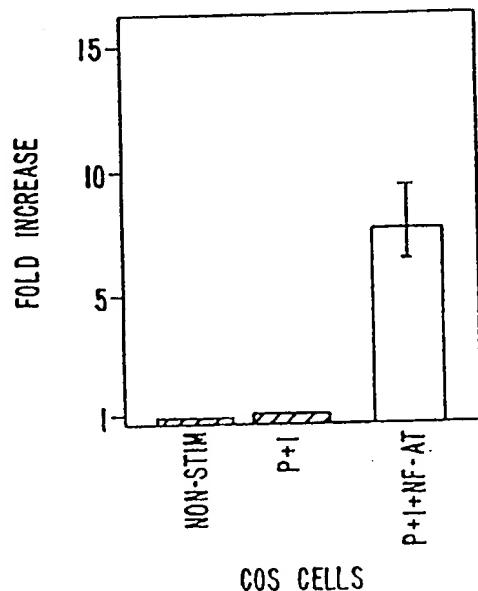


FIG. 6B.

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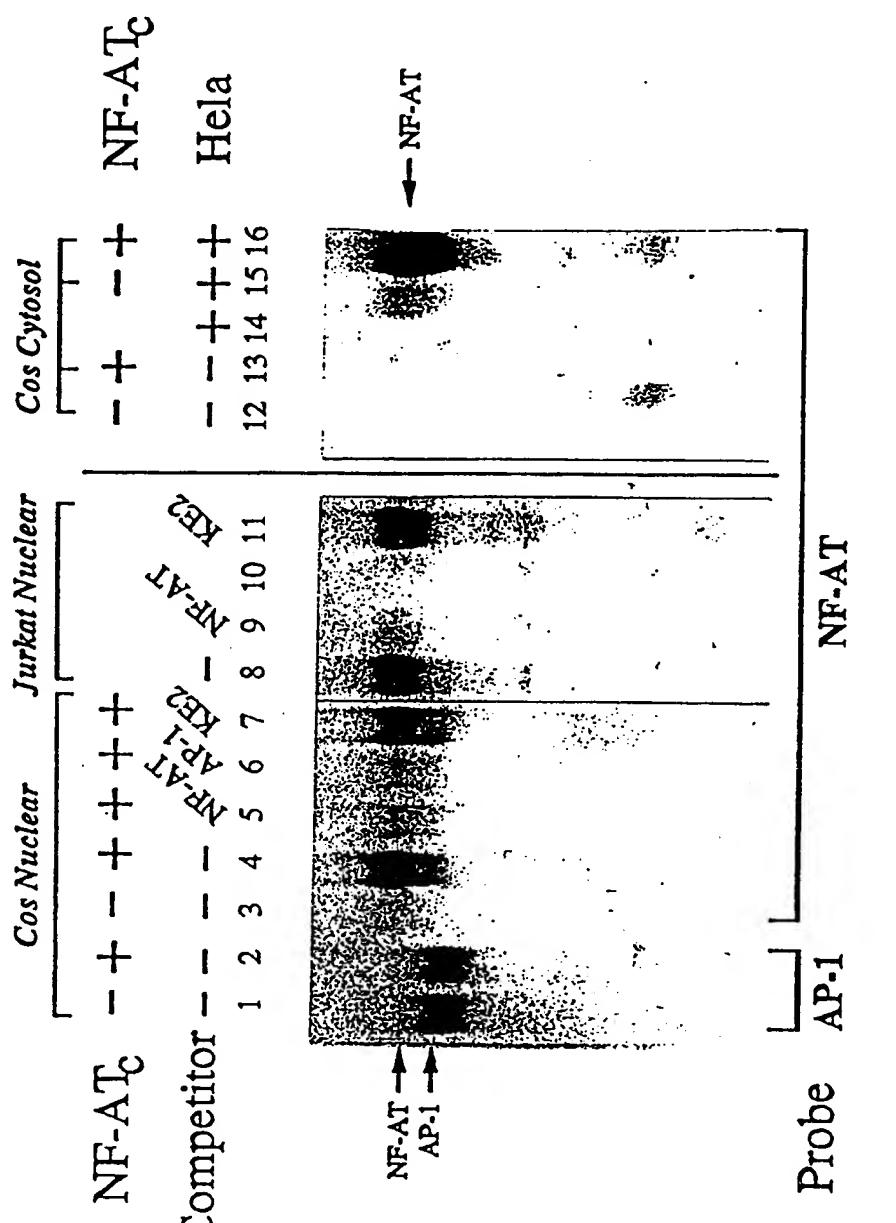


FIG. 6C.

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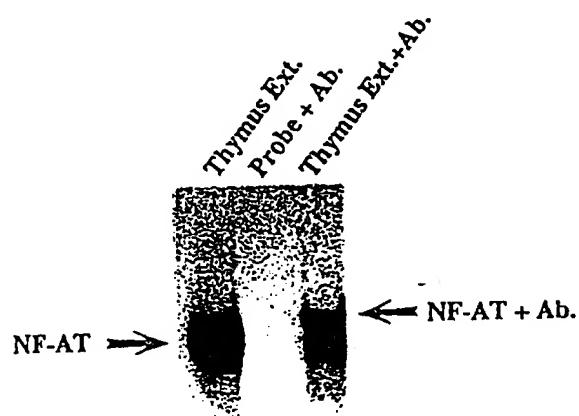


FIG. 6D.

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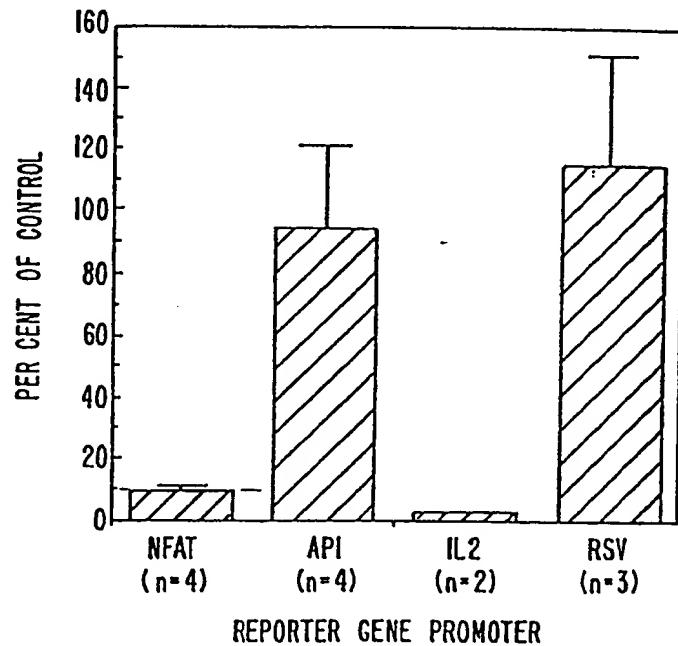


FIG. 7.

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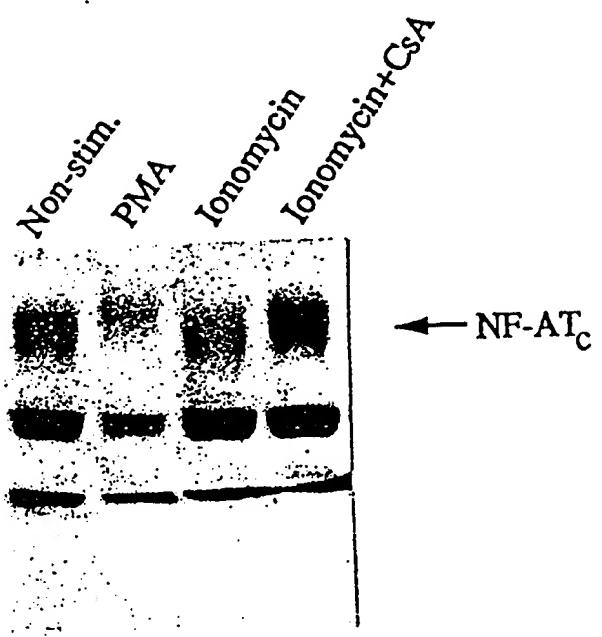


FIG. 8.

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FIG. 9A

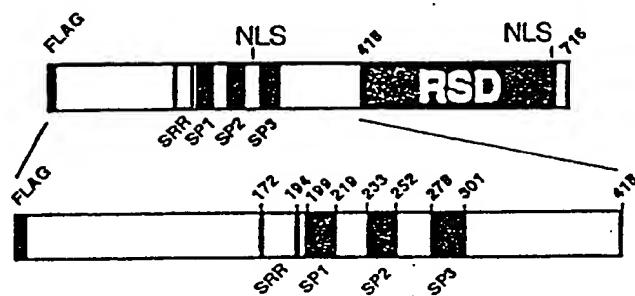


FIG. 9B

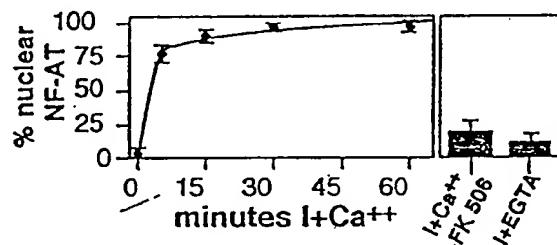
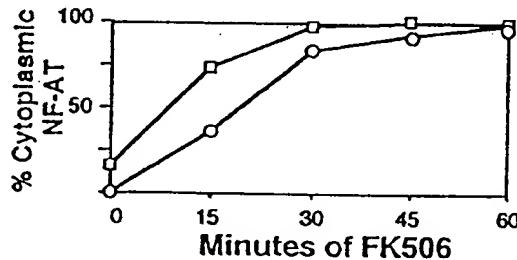


FIG. 9C



FIG. 9D



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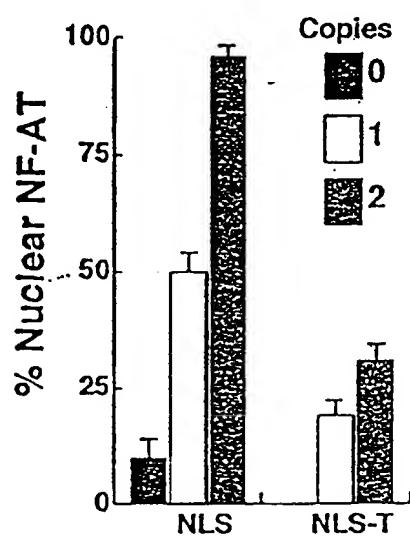


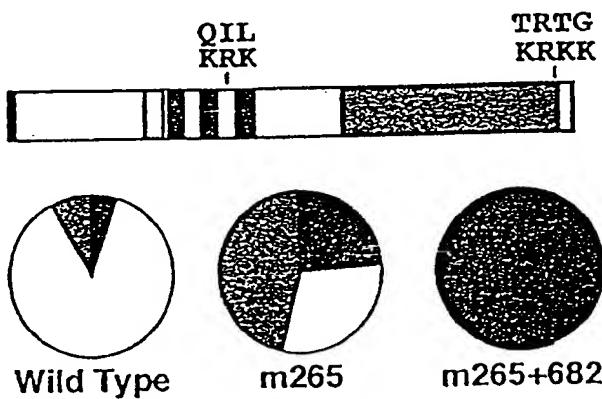
FIG. 10

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FIG. 11A

263-271 C N K R K Y S L N  
681-685 G K R K K

FIG. 11B

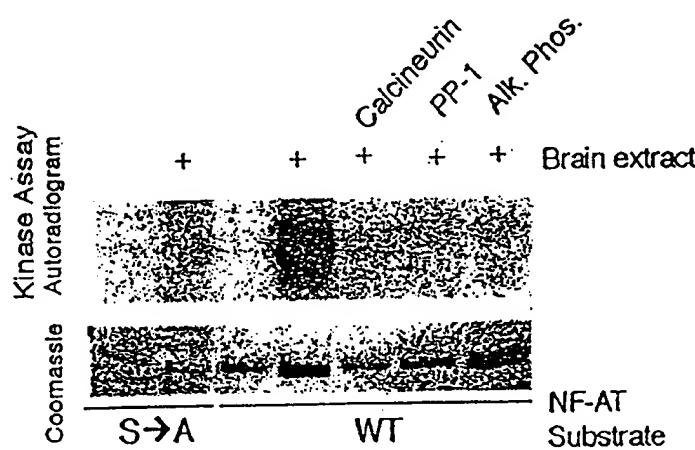


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FIG. 12A

|         | WT                        | CLSPASSLSSRSCNSEASSYESNYS | Localization |
|---------|---------------------------|---------------------------|--------------|
| mSRR    | --A--AA-AA-A--A--AA--A--A |                           | N            |
| 184-194 | -----A--AA--A--A          |                           | N            |
| 172-176 | --A--AA-----              |                           | N            |
| 178-181 | -----AA-A-----            |                           | N            |
| 184-188 | -----A--AA-----           |                           | N            |
| 191-194 | -----A--A                 | C                         |              |

FIG. 12B



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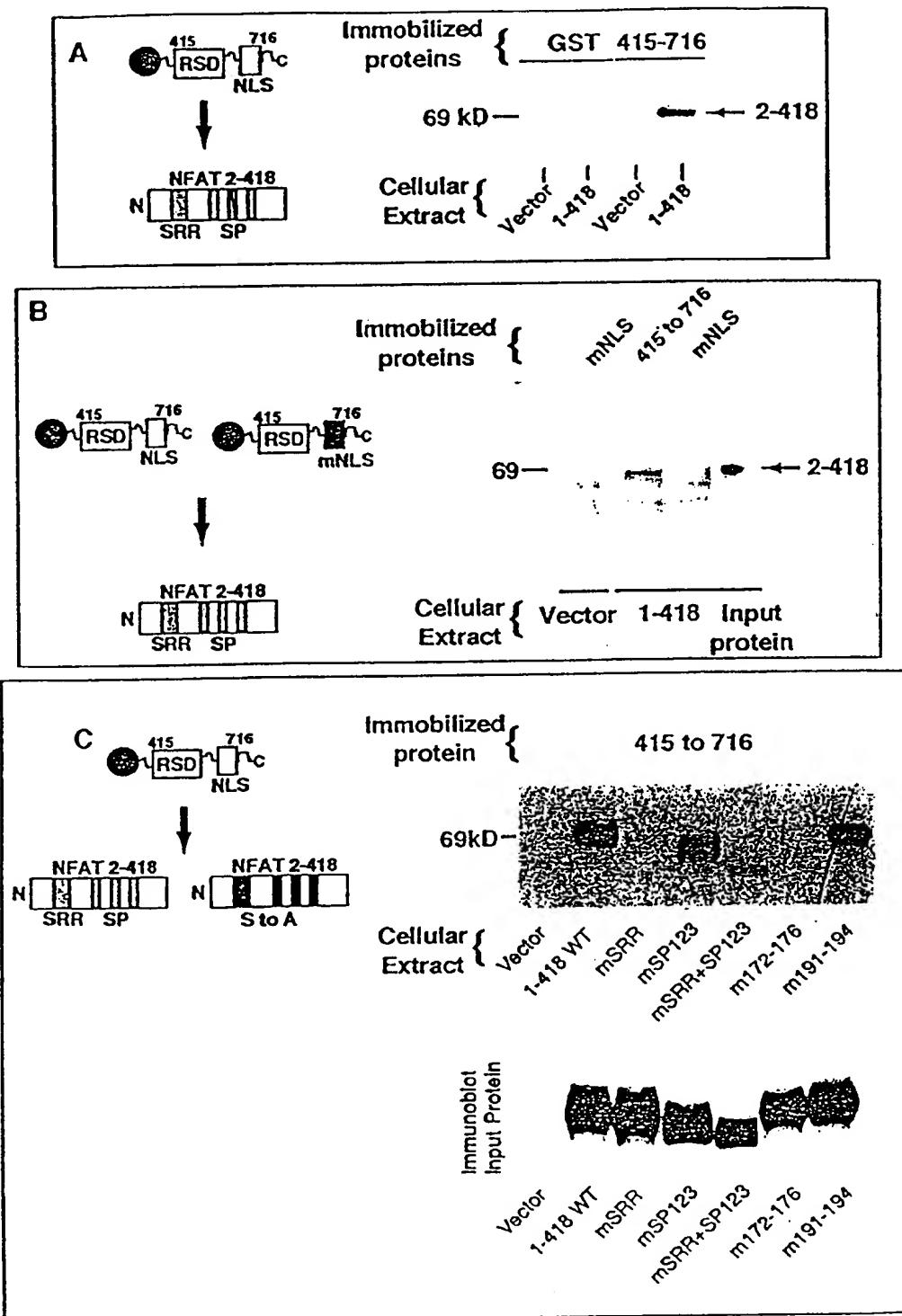


FIG. 13

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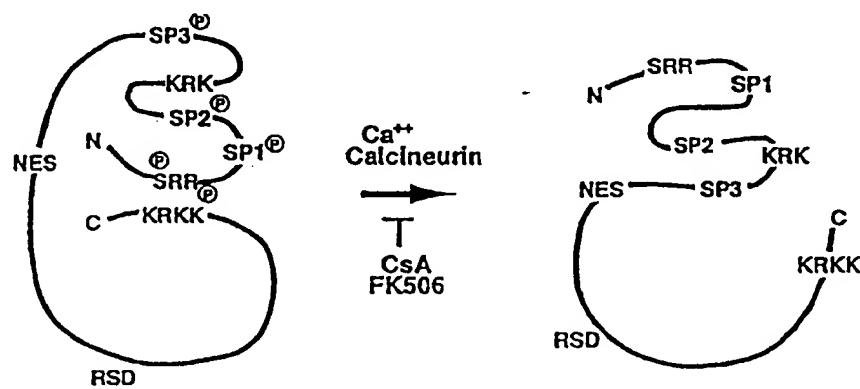


FIG. 14

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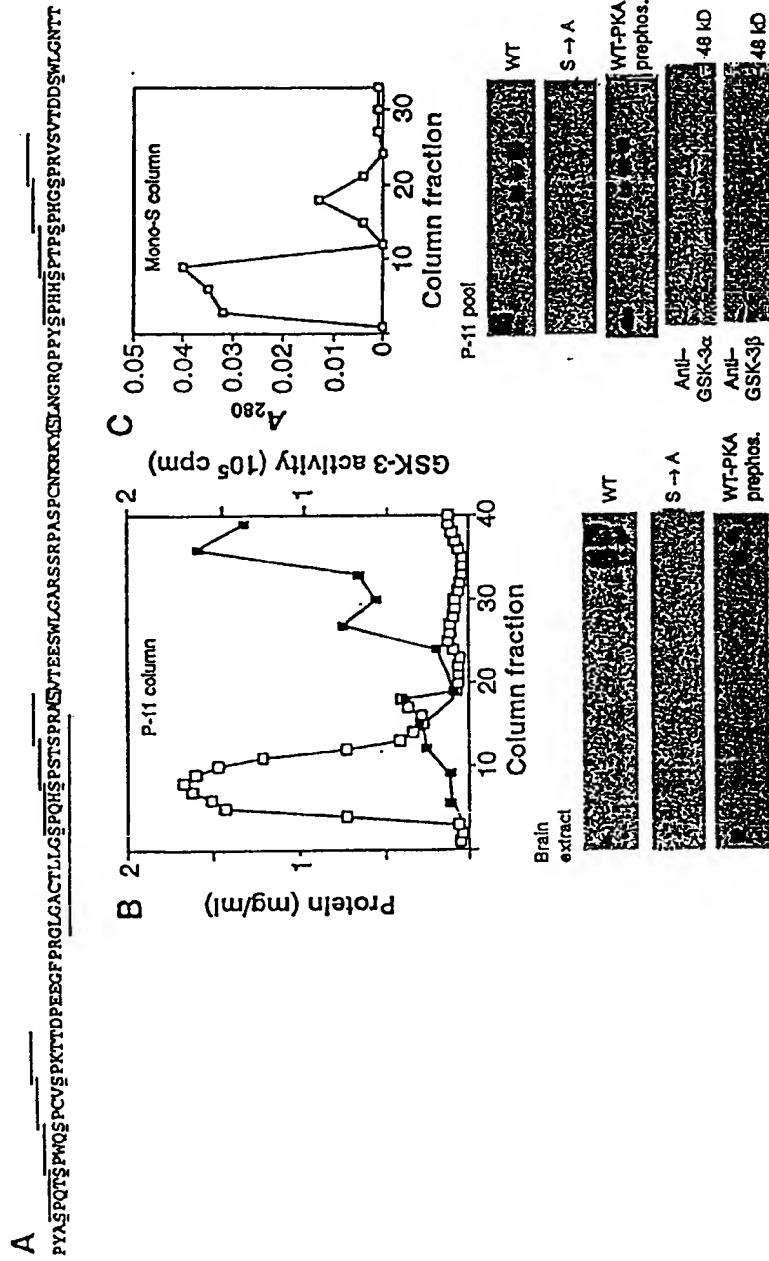


FIG. 15

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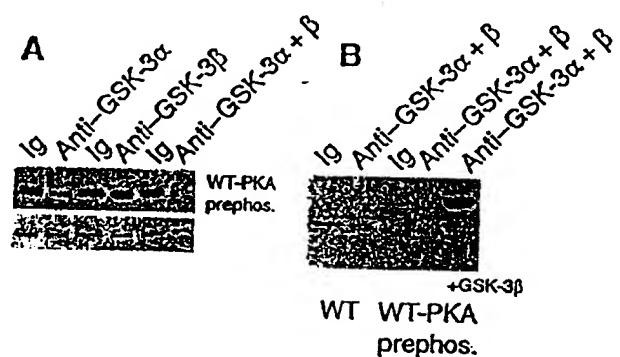


FIG. 16

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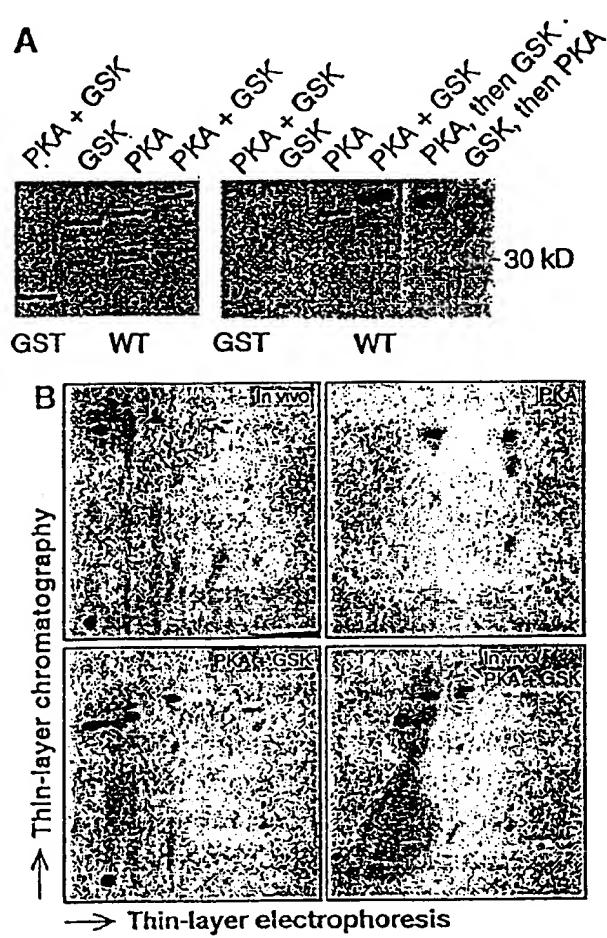


FIG. 17

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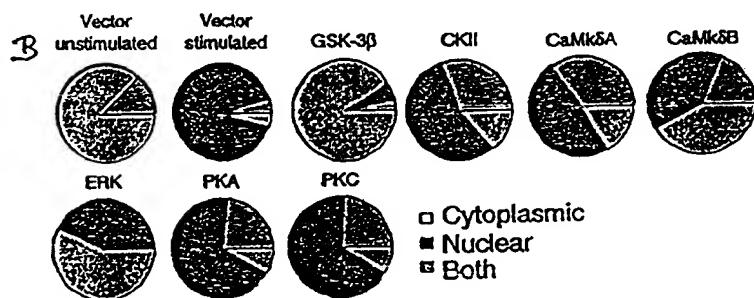
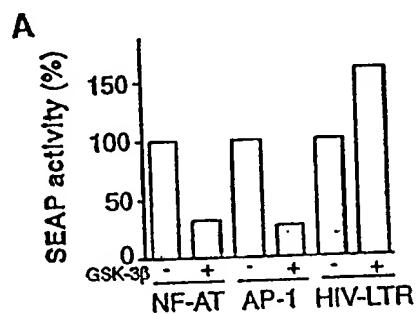


FIG. 18

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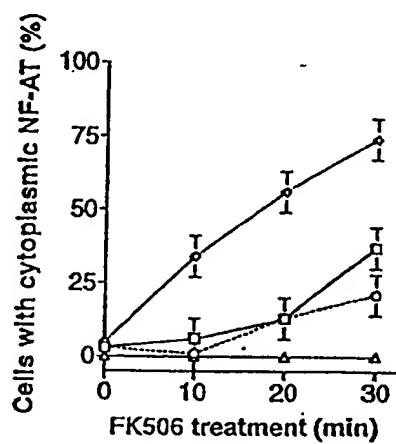


FIG. 19

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Figure 20A

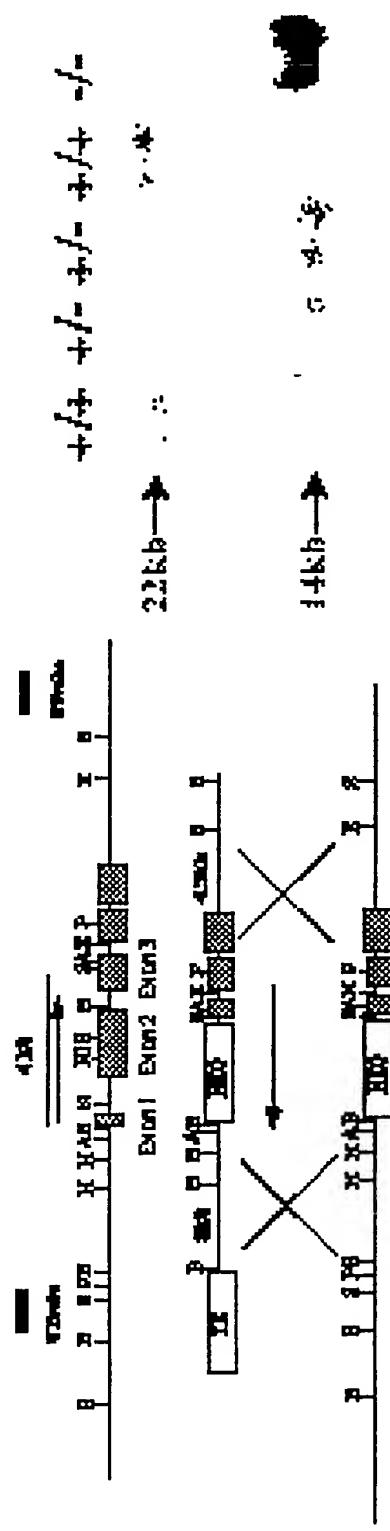


Figure 20B

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International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|  |  |  |  |
|--|--|--|--|
| <p>(51) International Patent Classification <sup>7</sup> :<br/>A61K 38/17, C07K 16/18, A61K 31/445, 31/70,<br/>A61P 9/00, 29/00, 35/00, 37/02 // C07K 14/57</p>  |  | A3   | <p>(11) International Publication Number: <b>WO 00/30671</b><br/>(43) International Publication Date: 2 June 2000 (02.06.00)</p> |
| <p>(21) International Application Number: PCT/US99/27862<br/>(22) International Filing Date: 23 November 1999 (23.11.99)</p>   |  | <p>(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> |  |
| <p>(30) Priority Data:<br/>09/198,977 24 November 1998 (24.11.98) US</p>   |  | <p>Published<br/><i>With international search report.</i></p>  |  |
| <p>(71) Applicant (<i>for all designated States except US</i>): THE BOARD OF TRUSTEES OF THE LELAND STANFORD JR. UNIVERSITY [US/US]; Stanford University, Stanford, CA 94305 (US).</p>   |  | <p>(88) Date of publication of the international search report:<br/>9 November 2000 (09.11.00)</p>                                   |  |
| <p>(72) Inventors; and<br/>(75) Inventors/Applicants (<i>for US only</i>): CRABTREE, Gerald, R. [US/US]; 7 Durham Road, Woodside, CA 94062 (US). NORTHRUP, Jeffrey, P. [US/US]; 313 North Milton Avenue, Campbell, CA 95008 (US). HO, Steffan, N. [US/US]; 3014 Award Row, San Diego, CA 92122 (US).</p> |  |  |  |
| <p>(74) Agents: VINCENT, Matthew, P. et al.; Foley, Hoag &amp; Eliot, LLP, One Post Office Square, Boston, MA 02109 (US).</p>  |  |  |  |

(54) Title: NF-AT MEDIATES CARDIAC HYPERTROPHY, METHODS AND REAGENTS RELATED THERETO

(57) Abstract

The invention is a method for treating cardiac hypertrophy, or other preventing other growth of cardiac and vascular tissue, through the use of NF-AT antagonists.

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Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/US 99/27862

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 7 A61K38/17 C07K16/18 A61K31/445 A61K31/70 A61P9/00  
 A61P29/00 A61P35/00 A61P37/02 //C07K14/57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|------------|---|-----------------------|
| X          | SUSSMAN M A ET AL: "Prevention of cardiac hypertrophy in mice by calcineurin inhibition."<br>SCIENCE,<br>vol. 281, no. 5383,<br>11 September 1998 (1998-09-11), pages<br>1690-1693, XP002140285<br>the whole document | 1-5,<br>15-33         |
| Y          | ---   | 6-14                  |
| P, X       | WO 99 19471 A (MOLKENTIN JEFFREY D ;GRANT STEPHEN R (US); OLSON ERIC N (US); UNIV)<br>22 April 1999 (1999-04-22)<br>page 1 -page 26, line 15<br>page 51 -page 57, line 2<br>---                                       | 1-5,8-33              |
|            | -/-   |                       |

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents

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Date of the actual completion of the international search

Date of mailing of the international search report

16 June 2000

06/07/2000

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**INTERNATIONAL SEARCH REPORT**

Internatinal Application No

PCT/US 99/27862

**C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|----------|--|-----------------------|
| Y        | BEALS C R ET AL: "Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3." SCIENCE, vol. 275, no. 5308, 28 March 1997 (1997-03-28), pages 1930-1933, XP002140286 the whole document                         | 6,7                   |
| Y        | WO 95 08554 A (UNIV LELAND STANFORD JUNIOR) 30 March 1995 (1995-03-30) page 8, line 7 -page 9, line 34 page 28, line 29 -page 45 claim 7; example 6  | 8-14                  |
| X        | claim 18   | 33                    |
| Y        | WO 96 26959 A (TULARIK INC) 6 September 1996 (1996-09-06) page 7, line 24 -page 10, line 9   | 8-14                  |
| A        | claim 10   | 20-27                 |
| X        | claims 1-8   | 33                    |
| Y        | WO 95 05389 A (UNIV LELAND STANFORD JUNIOR ;UNIV YALE (US); HARVARD COLLEGE (US)) 23 February 1995 (1995-02-23) page 6, line 7 -page 7, line 25  | 8                     |
| A        | claims 19-22   | 20-22,27              |
| X        | claims 1-7   | 33                    |
| A        | WO 94 15964 A (DANA FARBER CANCER INST INC ;HARVARD COLLEGE (US); RAO ANJANA (US)) 21 July 1994 (1994-07-21) cited in the application  | 20-30,32              |
|          | claims 17-21   |                       |
| A        | WO 93 04203 A (UNIV LELAND STANFORD JUNIOR) 4 March 1993 (1993-03-04)  | 28,31,32              |
|          | claims 19,28   |                       |
| A        | DE LA POMPA JL ET AL: "Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum." NATURE, vol. 392, no. 6672, 12 March 1998 (1998-03-12), pages 182-186, XP002140287 the whole document | 1-4,31                |
| P,X      | WO 99 40930 A (BLOOD RES CENTER) 19 August 1999 (1999-08-19) claims 106-109  | 33                    |

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-3, 15-18, 20, 23, 27, 28-33 all in part ; 4, 5, 26 in totality

Treatment of cardiac hypertrophy with inhibitors of NF-AT dephosphorylation.

2. Claims: 1-3, 15-18, 28-33 all in part ; 6, 7 in totality

Treatment of cardiac hypertrophy with stimulators of NF-AT phosphorylation.

3. Claims: 1, 2, 15-20, 23, 27, 28, 32, 33 all in part ; 8-11, 21, 22, 24, 25 in totality

Treatment of cardiac hypertrophy with inhibitors of NF-AT complex formation.

(It is assumed that claim 9 refers to claim 8, not to claim 7.)

4. Claims: 1, 2, 15-18, 28, 32, 33 all in part; 12-14 in totality

Treatment of cardiac hypertrophy with inhibitors of NF-AT production.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-33 relate to methods or compositions using compounds defined by reference to desirable characteristics or properties relative to NF-AT antagonism *sensu lato* (i.e. property to decrease NF-AT biological activity by any means as defined page 17, lines 15-23 of the description), namely inhibition of NF-AT dephosphorylation (Subject 1), stimulation of NF-AT phosphorylation (Subject 2), inhibition of the formation of complexes comprising NF-AT (Subject 3) and inhibition of NF-AT production (Subject 4).

The claims refer to all compounds having such characteristics or properties, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the used compounds by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to:

- calcineurin inhibitors, e.g. FK506 (examples 8, 9);
- GSK-3 overexpression (example 19);
- NF-AT mutants, e.g. the dominant negative mutant of example 6;
- NF-AT antisense nucleotides and ribozymes (page 68, lines 29-32 of the description);
- NF-AT antibodies (page 44, line 29 - page 49, line 32 of the description);
- and the generic concept of NF-AT antagonism in its broadest sense.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

**INTERNATIONAL SEARCH REPORT**

| Information on patent family members   |                  |                         |           | International Application No |
|--|------------------|-------------------------|-----------|------------------------------|
| Patent document cited in search report | Publication date | Patent family member(s) |           | Publication date             |
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|  |                  | JP                      | 6510189 T | 17-11-1994                   |
|  |                  | US                      | 5989810 A | 23-11-1999                   |
| WO 9940930 A                           | 19-08-1999       | NONE                    |           |                              |

**PENDING CLAIMS AFTER RESPONSE TO  
OFFICE ACTION DATED AUGUST 15, 2001**  
**UCSF-017CON2**

1. (Once Amended) An isolated macrophage of a mammal having integrated into its genome a DNA transcriptional controlling sequence positioned relative to a cell proliferation gene such that the presence of said DNA transcriptional controlling sequence and said cell proliferation gene results in macrophage proliferation.
2. The macrophage of claim 1, wherein said DNA transcriptional controlling sequence is a functional promoter sequence that increases expression of said cell proliferation gene.
3. The macrophage of claim 1, wherein said DNA transcriptional controlling sequence is a functional enhancer sequence that increases expression of said cell proliferation gene.
4. The macrophage of claim 1, wherein said cell proliferation gene is an oncogene.
5. The macrophage of claim 4, wherein said oncogene is selected from the group comprising *c-fes/fps*, *c-myc*, and *ras*.
6. The macrophage of claim 1, wherein said transcriptional controlling sequence is an enhancer/promoter sequence in an LTR of a human immunodeficiency virus.
9. (Once Amended) The method of claim 47, wherein said host is a mouse.
10. (Once Amended) The method of claim 47, wherein said tumor is in a tissue of said host selected from the group consisting of skin, peritoneum, and spleen.
44. (New) A method of screening for an agent that reduces macrophage viability, said method comprising:  
contacting a macrophage according to claim 1 with a candidate agent; and

determining the effect of said candidate agent on said macrophage, if any.

45. (New) The method of claim 44, wherein said screening is *in vitro*.

46. (New) The method of claim 44, wherein said screening is *in vivo*.

47. (New) A method of screening for an agent that reduces macrophage viability in a host, said method comprising:

contacting a macrophage according to claim 1 with a candidate agent in said host, wherein said host has a tumor comprising said macrophage; and

determining the effect of said candidate agent on said macrophage, if any.

48. (New) The method of claim 47, wherein the effect of said candidate agent on said tumor is determined.